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A METHOD OF ESTIMATING CURARE-LIKE ACTIVITY ON THE ISOLATED PHRENIC NERVE DIAPHRAGM PREPARATION OF THE RAT

BY

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(Received September 24 1946)

Preparations of curare have recently been introduced into medicine for two purposes. They have been used to reduce the force of the muscle contractions evoked in shock therapy of schizophrenia (Bennett, 1940, Cummins, 1942, Wolfe, 1945), they have also been used to obtain full muscular relaxation in anaesthesia, particularly in conjunction with cyclopropane (Griffith and Johnson, 1942, Mallinson, 1945, Gray and Halton, 1946) for operations in the upper part of the abdomen. The preparations used hitherto have been an extract of curare known as intocostin, prepared by E. R. Squibb and Sons, and *d*-tubocurarine chloride, the pure active principle isolated by King (1935) and prepared by Burroughs Wellcome and Co. The experiments described in this paper have been carried out with the latter.

As the supply of curare is limited there is a need for substitutes. At the same time a quick test method for comparison with curare is required. Previous workers have mostly used the isolated frog nerve-muscle preparation. The frog's gastrocnemius is less suitable than the sartorius muscle, because in the latter preparation the muscle is very thin and differences in diffusion rates of different compounds are negligible. Ing and Wright (1931) found the nerve sartorius preparation of *Rana esculenta* very suitable because the muscle recovered completely after poisoning, and consequently one preparation could be used to test several compounds. But if only small frogs (*Rana temporaria*) are available the nerve sartorius preparation is too delicate for routine tests. Also, most authors (for references, see Ing, 1936) have estimated either concentrations of drugs which paralysed the muscle completely or concentrations which just failed to cause complete paralysis. After such severe poisoning recovery is very slow, and routine tests would take a very long time. Testing curare extracts in Squibb's laboratories Holaday (quoted by Bennett, 1941) developed the head-drop method in the rabbit. Curare solutions are slowly infused intravenously until the animal is incapable of holding up its head. The amount per kg. necessary to produce this effect in rabbits is estimated. Results obtained by this method, together with those obtained on the acetylcholine contractions of the frog's gastrocnemius,

were claimed to be reproducible with an error of ± 1 per cent. The authors do not say whether several extracts could be compared in one animal or whether several rabbits were used for each estimation. Also, it seems to be more desirable to use a method with a clearer endpoint.

Recently Bulbring (1946) described a mammalian isolated nerve-muscle preparation, the rat's phrenic nerve-diaphragm, which has several advantages: the rat's diaphragm is a thin muscle which gives constant contractions for many hours and allows drugs which are applied to it to be washed out readily. A method of estimating curare-like substances has been worked out using this preparation.

METHOD

The rat's phrenic nerve-diaphragm preparation was set up as described by Bulbring (1946). The fan shaped muscle strip was stimulated indirectly to maximal contractions by condenser discharges from a neon-lamp circuit. The contractions of the muscle were recorded by an isotonic lever. The muscle was immersed in a bath containing Tyrode solution with twice the normal amount of glucose. Pure oxygen or a mixture of 95 per cent oxygen and 5 per cent CO_2 was supplied in fine bubbles by means of a gas distribution tube. The bath was kept at a constant temperature between $37\text{--}38^\circ\text{C}$. Its capacity was 100 ml. Between the addition of one dose of tubocurarine and the next there was an interval of 13 minutes during which the Tyrode solution was changed four times and the regular stimulation of the muscle was not interrupted. The routine procedure was as follows: a dose of tubocurarine was added to the bath and allowed to act for 3 min. after which the Tyrode solution was changed, 2, 4, and 6 min. later the solution was changed again, after which the preparation was left for 5 min. before adding the next dose of tubocurarine. By keeping to this schedule it was possible to obtain a satisfactory recovery of muscle contractions and a constant response to a given dose of tubocurarine, provided a dose was chosen which did not produce a complete abolition of muscle contractions but only a partial inhibition, preferably of not more than 50 per cent.

The percentage inhibition was determined by measuring the height of contraction just before tubocurarine was added to the bath, and again 3 min. later. Thus if the first figure was 76 mm. and the second figure was 58 mm., then the contraction was reduced to 76.5 per cent of its original height, and the inhibition was said to be 23.5 per cent.

The effect of the rate of stimulation—There are very few references in the literature to any relation between the effect of curare and the rate of stimulation. In 1935 Briscoe, using the quadriceps muscle of the decerebrate cat, found that the action of curare in diminishing rigidity was greater when the stimulation was fast than when it was slow. It was easy to determine the effect of different frequencies of stimulation on the action of tubocurarine on the isolated preparation. The response to a given dose of tubocurarine was almost constant, except for the response to the first dose applied to a fresh preparation, or to a preparation left without tubocurarine for an interval, such a response was less than later responses. It was found that an increase in rate of stimulation correspondingly increased the action of tubocurarine (Fig. 1). At a rate of 5 per min., the addition of 0.09 mg. *d*-tubocurarine chloride diminished the muscle contractions in 3 min. by 11.5 per cent while at a rate of 20 per min. the diminution was

36.5 per cent. No experiment was done at a rate greater than 20 per min because, presumably owing to fatigue, the muscle then recovered very slowly from the effect of the tubocurarine. A quantitative study of the relation between rate and effect was made in different experiments, and the results of two experiments are given in Fig 2, which shows the relation between the logarithm of the concen-

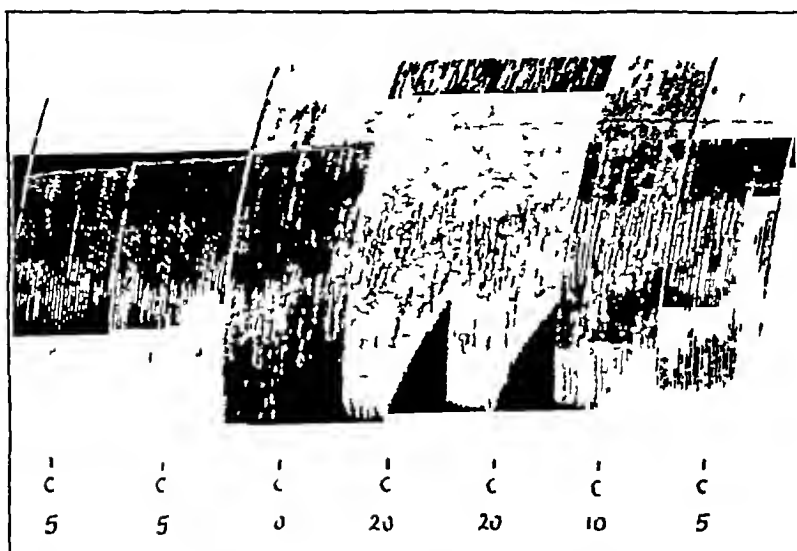


FIG 1—Rat Isolated diaphragm. Record of muscle contractions in response to maximal stimulation of the phrenic nerve. Contractions downwards. Effects of constant doses of 90 μ g. *d*-tubocurarine chloride (C) at different rates of stimulation indicated below.

tration of tubocurarine and the percentage inhibition of muscle contractions. In these experiments a diminution in the rate of stimulation from 20 to 5 per min required, in order to obtain the same effect, that the dose of tubocurarine should be increased 1.2 times. It was also observed that when the stimulation was stopped for 5 min at the moment of adding tubocurarine, and then restarted for 3 min, the reduction of contractions in this period was less than when the stimulation was continued for 8 min without interruption. It is evident that the effect produced by tubocurarine is greater when the muscle is working and that it is a function of the rate of stimulation. A variation in the strength of stimuli had no influence on the action of tubocurarine.

The effect of carbon dioxide—The curare action was less when pure oxygen was used than when the gas mixture consisted of 95 per cent oxygen and 5 per cent carbon dioxide (Fig 3). The action of tubocurarine in the presence of 5 per cent CO_2 was about 2.5 times that in the presence of pure oxygen. Thus

the inhibition produced by 0.1 mg *d*-tubocurarine chloride in 100 ml was about 30.5 per cent with oxygen, but 74 per cent with a mixture of oxygen and CO_2 . This increased action of tubocurarine by the gas mixture is most likely due to the action of CO_2 , as Jacobs and Stewart (1942) have found that CO_2 catalyses

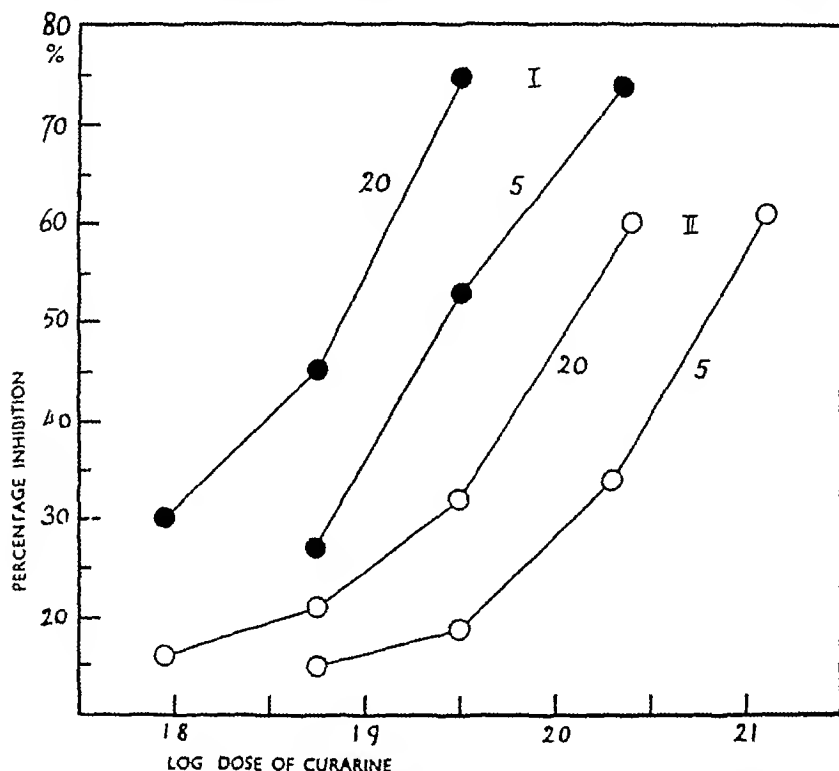


FIG 2—Isolated phrenic nerve diaphragm of the rat. Abscissae=logarithm of tubocurarine concentration. Ordinates=percentage inhibition of muscle contractions. The effect of different rates of stimulation (20 and 5 per min) on the action of tubocurarine is shown in two experiments (I and II).

the diffusion of ions through membranes. The pH of the fluid in the bath, determined by an indicator method, was 8.8 when oxygen was used, and 7.6 when the mixture of oxygen and CO_2 was turned on.

Estimation of unknown samples of tubocurarine—The quantitative study of a drug such as tubocurarine, which causes cessation of some physiological process which is not well understood, is often fraught with difficulties, this is especially true when the drug is not rapidly destroyed in the tissue and consequently continues to exert its action if it is not removed either by the circulation as *in vivo* or by washing as *in vitro*. However, the isolated rat's nerve diaphragm

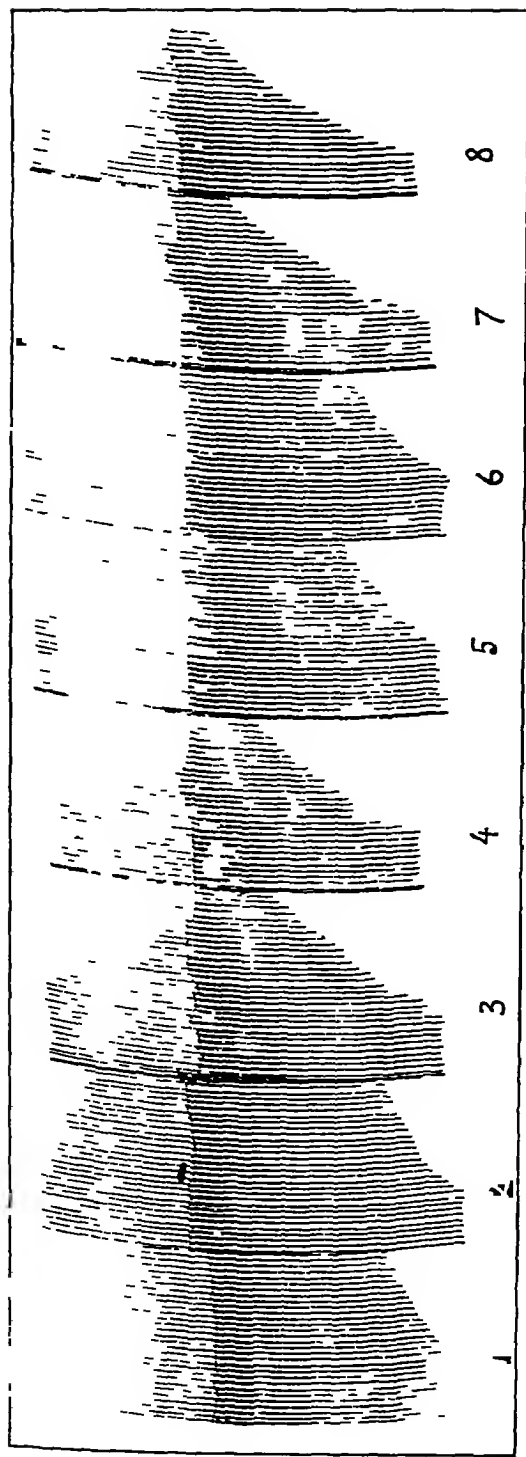


FIG 3—Record as Fig 1. A constant dose of 100 μ g *d*-tubocurarine chloride was added at 1, 2, 5, and 6 during oxygenation with pure O_2 , at 3, 4, 7, and 8 during oxygenation with 95 per cent O_2 and 5 per cent CO_2 .

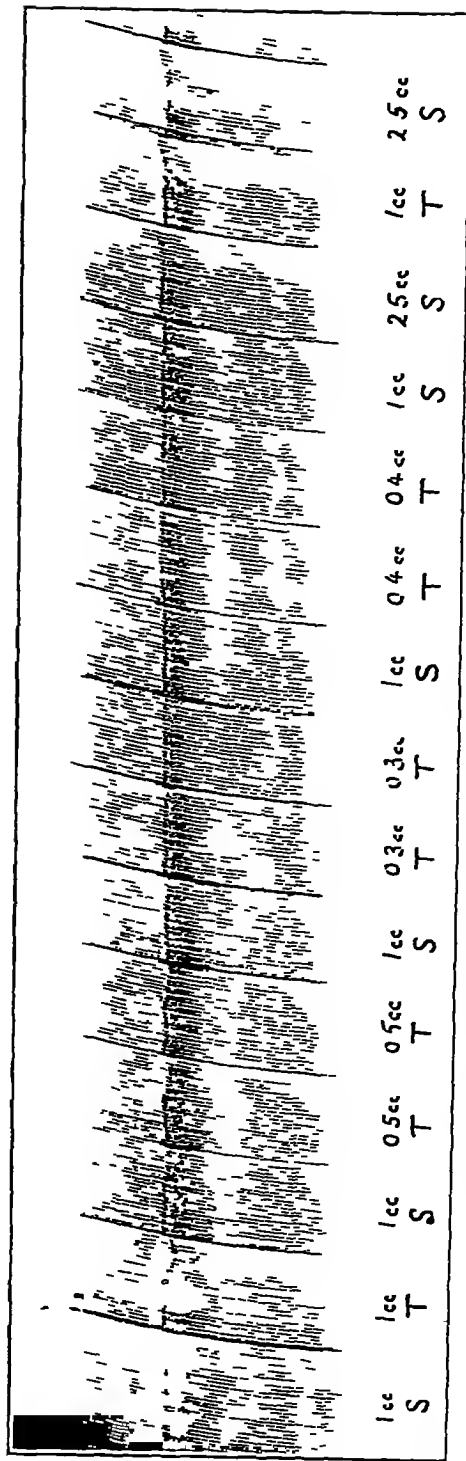


FIG 4—Record as Fig 1. Illustration of the estimation of an unknown tubocurarine solution (T) by comparison with a known solution (S). For detailed description see text.

preparation gives results which are quite reproducible, in many preparations as many as 36 doses could be tested. The results in Table I show the regularity with which tubocurarine was found to exert its action in five different experiments. In these the rate of stimulation was 5 per min.

TABLE I

Concentration of <i>d</i> -tubocurarine chloride $\mu\text{g. per ml}$	Percentage inhibition					
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Mean \pm S.E.
1.0	14	8	13	15	13	12.6 ± 1.2
1.4	30	19	30	39	32	30.0 ± 3.2
1.8	42	44	54	57	49	49.2 ± 2.5
2.0	62	61	67	70	67	65.4 ± 1.7

In ten experiments an unknown solution of tubocurarine was estimated by comparison with a known solution. The rate of stimulation in the first two experiments was 20 per min., in the other eight it was 5 per min. Each time the effect of equal doses was observed at first and then the effects of the test solution (T) and the standard solution (S) were bracketed as closely as possible. Fig. 4 is an example of the procedure. 1 ml S was weaker than 1 ml T. 0.5 ml T was stronger, 0.3 ml T was weaker than 1 ml S, 0.4 ml T

TABLE II

ESTIMATION OF UNKNOWN SOLUTIONS OF *d*-TUBOCURARINE CHLORIDE (T) BY COMPARISON WITH A STANDARD SOLUTION (S) CONTAINING 200 $\mu\text{g. per ml}$

Date	Rate of Stimulation per min	Effect of equal doses	Bracket effect ml T > or < ml S	Geometrical mean of T equivalent to 1 ml S	Observed value ($\mu\text{g. per ml}$)	Actual value ($\mu\text{g. per ml}$)	Percentage error
30 Nov '45	20	T < S	0.77 > 0.45 0.54 < 0.45	1.42	141	150	- 6.0
3 Dec. '45	20	T = S	—	—	200	220	-10.0
2 Sept '46	5	T < S	1.4 > 1 1.0 < 1	1.18	169.5	166	+ 2.1
3 Sept '46	5	T > S	0.8 > 1 0.5 < 1	0.63	317	333	- 4.8
4 Sept '46	5	T < S	1.3 > 1 1.0 < 1	1.14	175.5	180	- 2.5
5 Sept. '46	5	T > S	0.5 > 1 0.3 < 1	0.39	513	500	+ 2.6
5 Sept '46	5	T < S	1 < 1 1.2 > 1	1.09	183.5	200	- 8.8
6 Sept. '46	5	T > S	0.5 > 1 0.3 < 1	0.39	513	475	+ 8.0
9 Sept '46	5	T > S	0.5 > 1 0.66 > 1	0.57	350	380	- 7.1
10 Sept '46	5	T > S	0.6 < 1 0.48 > 0.6	0.69	290	270	+ 6.9

was equal to 1 ml S, similarly, 1 ml T was equal to 2.5 ml S. As S contained 200 μ g. curarine per ml T was estimated to contain 500 μ g., actually it contained 475 μ g. per ml. Not all experiments were so accurate that the arithmetical mean of the bracketing results could be used and it was found that the geometrical mean usually gave the better result. Table II shows the values obtained in all the experiments treated in this way.

DISCUSSION

The use of the rat's phrenic nerve-diaphragm preparation for the estimation of curare-like activity offers several advantages over current methods. It is an isolated mammalian preparation which is sufficiently thin to allow substances to exert their action quickly and also to be washed out readily. It is therefore possible to observe an effect within 3 min and, if an action has taken place, to observe another effect 10 min later. As the preparation remains in good condition for many hours and doses can be administered at 13 min intervals, a large number of estimations can be made in one day.

SUMMARY

A method is described for estimating curare-like activity using the isolated phrenic nerve-diaphragm preparation of the rat. The preparation is easily set up, results are readily reproduced over many hours, and the error of the method is about 6 per cent.

I wish to thank Prof. Burn and Dr. Bülbring for their encouragement and guidance in this work.

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THE RELATIVE ACTIVITY OF PROSTIGMINE HOMOLOGUES AND OTHER SUBSTANCES AS ANTAGONISTS TO TUBOCURARINE

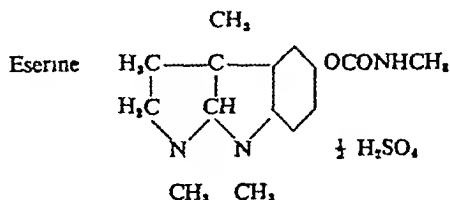
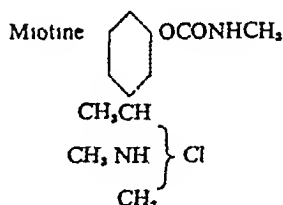
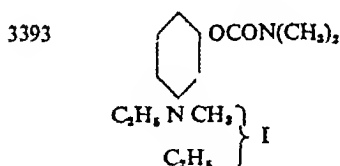
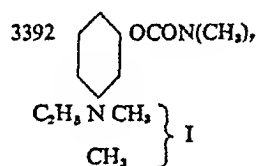
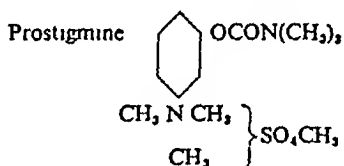
BY

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From the Department of Pharmacology, Oxford

(Received September 24 1946)

The need for substances which antagonize curare has arisen with the recent introduction of the drug into medicine. There is a serious danger of giving too much and causing respiratory failure. While it is true that this occurrence will usually demand artificial respiration, the length of time for which it must be continued can be greatly reduced by the injection of a suitable antagonist. The substances investigated have been the following:



In addition, guanidine and dimethyl guanidine have been tested, since guanidine is stated by Minot, Dodd, and Riven (1939, 1941) to be a more useful substance for treating myasthenia than prostigmine itself.

The first series of experiments was made on the isolated nerve-muscle preparation of the rat, recently described by one of us (Bulbring, 1946). Experiments

were then performed on the whole animal to see whether the relationships observed in the isolated nerve-muscle preparation held also for muscle in its natural surroundings. Some toxicity tests were made on mice in order to compare the therapeutic indexes of the compounds. Finally, experiments were carried out to determine the relative anticholinesterase action of the prostigmine homologues in the Warburg apparatus.

EXPERIMENTAL RESULTS

1 *The isolated phrenic nerve-diaphragm preparation of the rat*

The method has been described in detail by Büllbring (1946) and also in the preceding paper by Chou. The isolated strip of diaphragm was stimulated through the phrenic nerve by maximal single shocks at a constant rate of 5 per min. The procedure followed was first to determine the percentage inhibition produced by tubocurarine alone within a standard time of 3 min., the height of the muscle contraction was measured just before tubocurarine was added and also the height of contraction at the end of 3 min., during which tubocurarine was present. The percentage diminution was calculated from these two figures. Between one dose of tubocurarine and the next there was an interval of 15 min during which the

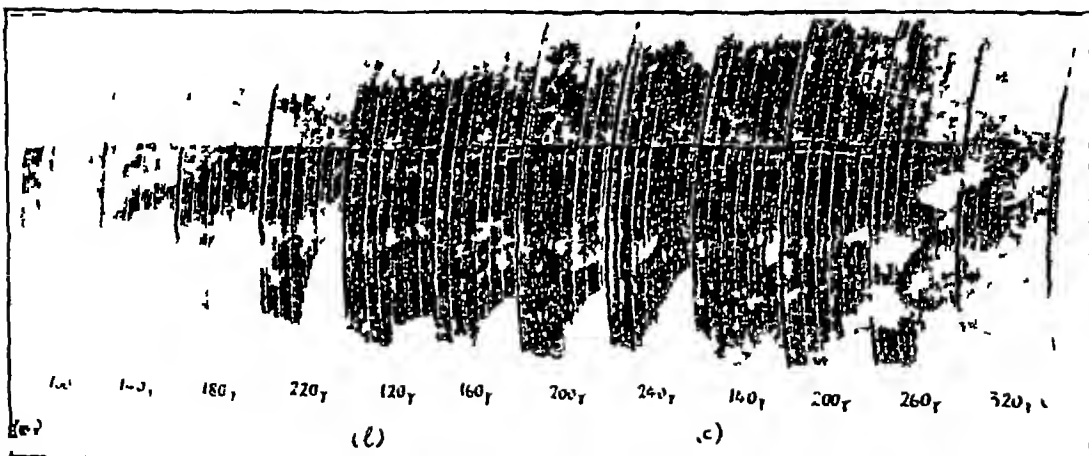


FIG 1—Rat. Isolated phrenic nerve-diaphragm preparation. Record of muscle contractions (contractions downwards) rate of stimulation 5 per min. The inhibition caused by four increasing doses of tubocurarine alone (a) is compared with that of four larger doses in the presence of the antagonist 3392 (b) in a concentration of 5×10^{-8} , and (c) in a concentration of 10^{-8} .

fluid in the bath was changed four times as described in the preceding paper for the estimation of tubocurarine. At the beginning of each experiment three or four concentrations of tubocurarine alone were tested. Next the antagonist was added to the bath 1 min. before tubocurarine was added and the inhibition which the latter produced in 3 min. was determined. An example is given in Fig. 1. The first portion of the record shows the action of four increasing concentrations of tubocurarine alone while the later portion shows the action of four greater concentrations in the presence of a fixed concentration of the

prostigmine homologue 3392. An attempt was made to match the inhibitions produced by tubocurarine alone with similar inhibitions when both tubocurarine and the antagonist were present together. This attempt was made not only for one concentration of the antagonist but for three or four.

The results were plotted on a graph, as shown in Fig 2, where the abscissae are the log doses of tubocurarine and the ordinates are the percentage inhibitions. Curve A shows the relation when tubocurarine alone was used, curve B

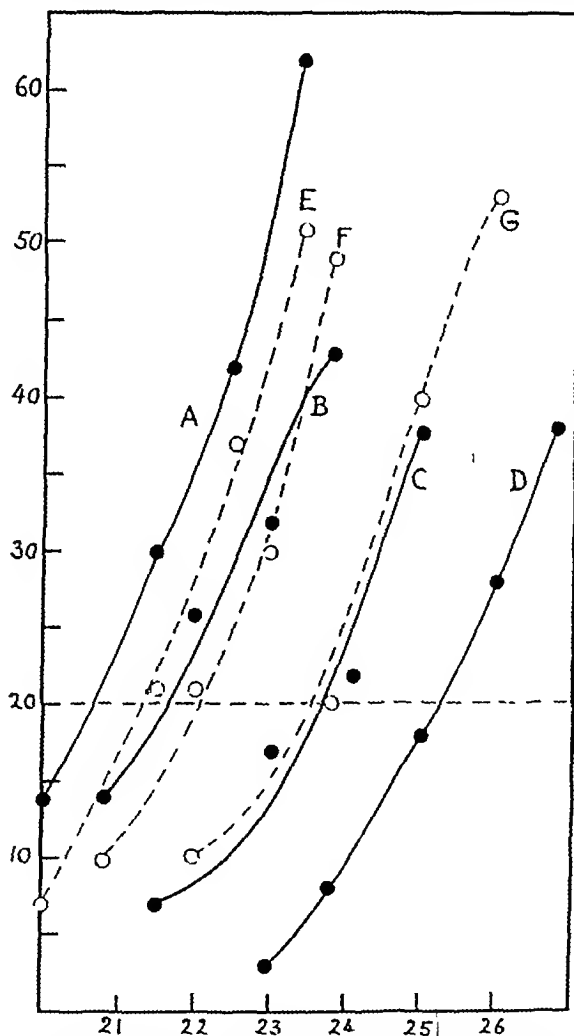


FIG 2—Assay of anti-curare activity. Abscissae=log dose of tubocurarine. Ordinates=percentage inhibition. The dose of tubocurarine causing 20 per cent inhibition can be estimated for tubocurarine alone (A). It has to be increased with increasing concentrations of antagonists—i.e., B= 5×10^{-5} 3392, C= 10^{-5} 3392, D= 3×10^{-4} 3392. E, F, and G represent results with corresponding concentrations of prostigmine, which is shown to be weaker than 3392.

TABLE I
COMPARISON OF TUBOCURARINE ANTAGONISTS

Date	Concentration of antagonist	Dose of tubocurarine (μ g.) causing 20 per cent inhibition in the presence of		Ratio
		Eserine	Prostigmine	
7 & 10 Dec.	10^{-8}	126	237	0.53
1 & 2 Jan.	10^{-8}	100	114	0.88
9 Jan.	10^{-8}	108	95	1.14
	2×10^{-8}	—	108	
	5×10^{-8}	197	—	
10 Jan	5×10^{-8}	69	78	0.89
	10^{-8}	91	106	0.86
	3×10^{-8}	124	143	0.87
11 Jan	5×10^{-8}	96	89	1.08
	10^{-8}	84	130	0.65
	2×10^{-8}	93	132	0.70
		Mean \pm S.E.		0.85 \pm 0.06
		Miotine	Prostigmine	
19 Feb	5×10^{-9}	120	117	1.03
	10^{-8}	134	128	1.05
7 March	5×10^{-8}	125	97	1.29
	10^{-8}	199	151	1.32
	3×10^{-8}	215	160	1.35
		Mean \pm S.E.		1.21 \pm 0.07
		3392	Prostigmine	
26 Feb	5×10^{-8}	140	134	1.05
	10^{-8}	228	159	1.54
	3×10^{-8}	326	223	1.46
6 March	5×10^{-8}	143	127	1.13
	10^{-8}	226	141	1.61
	3×10^{-8}	393	200	1.97
		Mean \pm S.E.		1.46 \pm 0.13
		3393	Prostigmine	
7 Feb	5×10^{-9}	178	145	1.22
	10^{-8}	224	131	1.71
	3×10^{-8}	305	184	1.66
8 Feb	5×10^{-8}	320	182	1.76
	10^{-8}	—	221	—
	3×10^{-8}	—	321	—
11 Feb	5×10^{-8}	192	158	1.22
	10^{-8}	312	173	1.86
	3×10^{-8}	416	251	1.66
5 March	5×10^{-8}	257	149	1.74
	10^{-8}	319	162	1.97
	3×10^{-8}	398	184	2.16
12 March	2×10^{-8}	143	—	—
	5×10^{-8}	—	127	—
	10^{-8}	—	162	—
		Mean \pm S.E.		1.70 \pm 0.09

shows the relation in the presence of 3392 in a concentration of 5×10^{-9} , curve C shows the relation when the concentration of 3392 was 10^{-8} , and finally curve D shows the relation when the concentration was 3×10^{-8} .

To make the comparison between one antagonist and another, for example between 3392 and prostigmine, further observations were then made on the same preparation, in which the effect of different concentrations of tubocurarine was observed in the presence of each of a series of concentrations of prostigmine. Results obtained in this way are shown in Fig 2 as curves E, F, and G. It was found better to arrive at the relative potency of two antagonists by considering only results obtained on the same preparation rather than to compare the effect of one antagonist in one preparation with that of another in a second preparation.

Quantitative expression of results—From the graph shown in Fig 2 it was possible to determine the concentration of tubocurarine which caused 20 per cent inhibition in the presence of each of the different concentrations of antagonist.

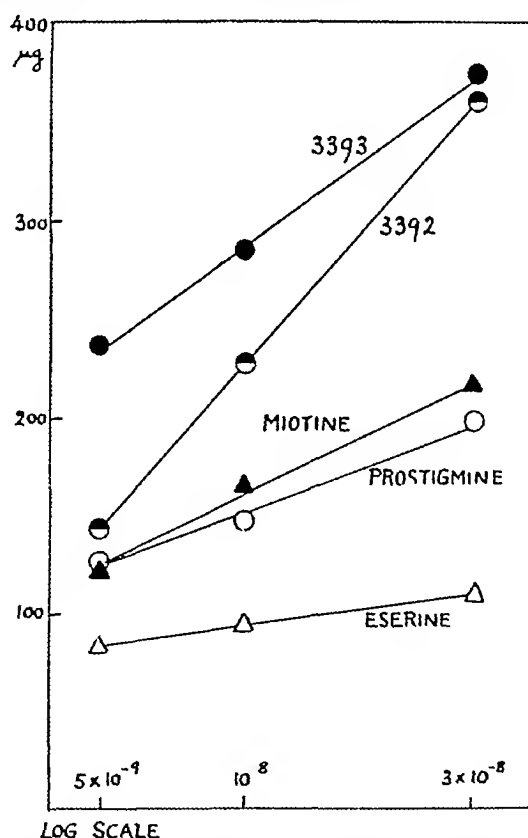


FIG 3—The relation between the concentration of antagonist and the dose of tubocurarine required to produce 20 per cent inhibition. Abscissae=log concentration of antagonist. Ordinates=dose of tubocurarine causing 20 per cent inhibition (100 ml bath)

An expression for the anti-curarine activity of the substances investigated was then obtained, using prostigmine as a standard. The results in Table I represent the experiments in which the different substances were compared.

The ratio of the amount of tubocurarine required to reduce the muscle response by 20 per cent varied little if it was calculated in the presence of the same concentration of each antagonist. If, however, an attempt was made to bracket the effective dose of curarine in the presence of one concentration of an antagonist between two doses in the presence of different concentrations of a second antagonist, the results obtained varied widely. The reason for this difficulty is obvious from Fig 3, in which all the results obtained were combined, the graph shows that the anti-curare activity of the different substances is raised by different degrees as their concentration is increased. It rises more steeply for 3392 than for prostigmine and less steeply for eserine. Thus an increase in the concentration of the antagonist from 5×10^{-9} to 3×10^{-8} requires 57 per cent increase of the dose of tubocurarine in the presence of prostigmine, in the presence of eserine only 32 per cent increase is required, while in the presence of 3392 the dose of tubocurarine has to be increased by 155 per cent.

The general results obtained are set out in Table II, in which prostigmine is given the value 1, the ratios are derived from the data given in Table I.

TABLE II

ANTI-CURARE ACTIVITY RELATIVE TO PROSTIGMINE = 1

Eserine 0.85	3392 1.46
Miotine 1.21	3393 1.70

The interesting result was thus obtained that substitution of the methyl groups attached to the N atom of the basic radical in the prostigmine molecule, first by one ethyl group and then by a second, led to an increase in anti-curare action.

Another comparison was made between eserine, prostigmine, miotine, and 3393, in which tubocurarine and its antagonist were added to the bath in doses which were the same throughout, viz., 0.4 mg tubocurarine chloride and 1 μ g of the antagonist (100 ml bath). The degree of inhibition of the muscle contraction was determined after 3 min exposure to tubocurarine when tubocurarine and antagonist were added simultaneously and also when the antagonist was added 3 min, 6 min, and 12 min before the tubocurarine. The results obtained are shown in Fig 4 from which it will be seen that there was little difference between eserine and prostigmine; these, however, were weaker than miotine and 3392, which were also similar in activity to one another. Miotine and 3392 were in turn definitely weaker than 3393, which was easily the strongest substance investigated.

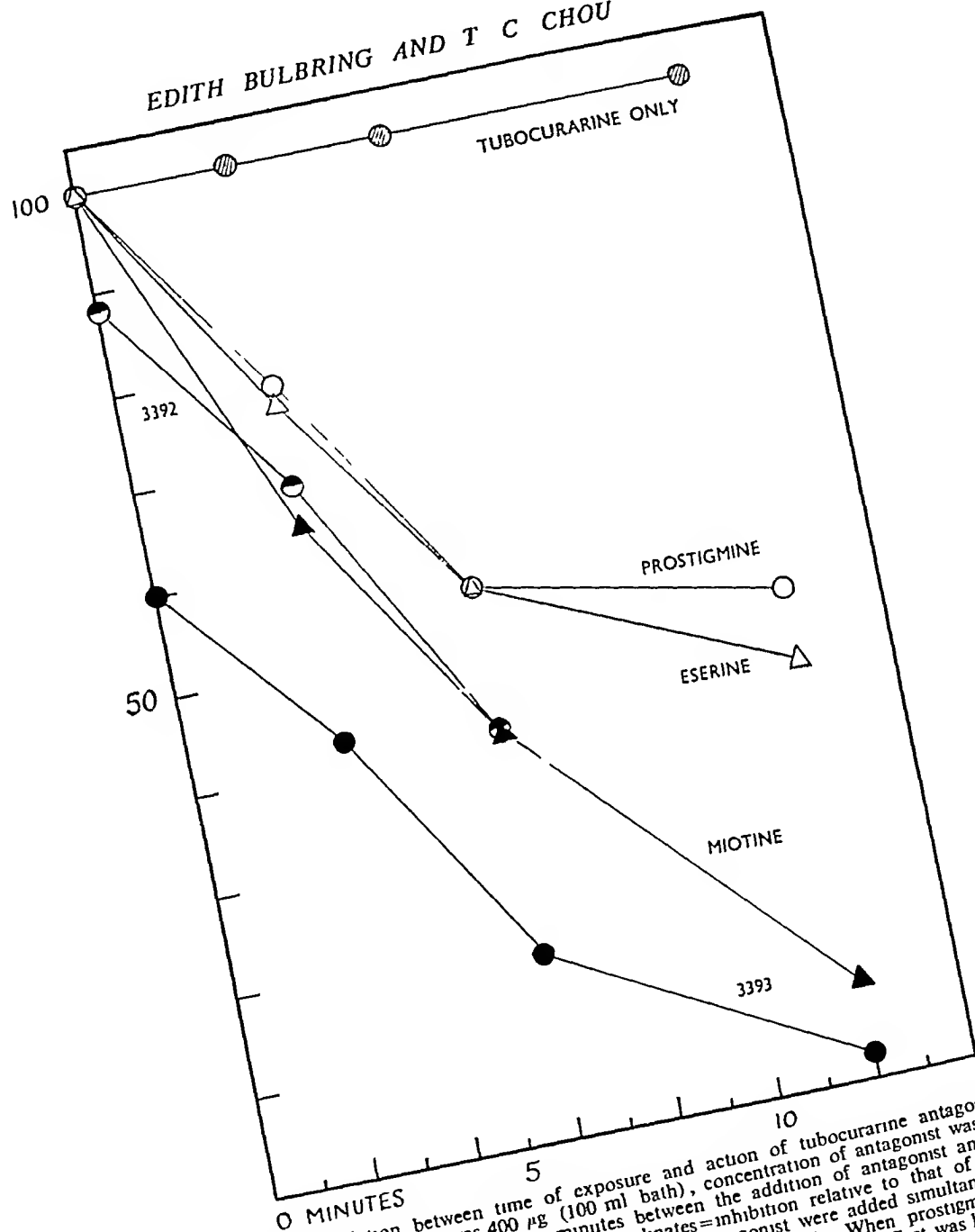


FIG 4—The relation between time of exposure and action of tubocurarine antagonists. Dose of tubocurarine was 400 μ g (100 ml bath), concentration of antagonist was 10^{-4} throughout. Abscissae=time in minutes between the addition of antagonist and the subsequent addition of tubocurarine. Ordinates=inhibition relative to that of tubocurarine alone as 100. When tubocurarine and antagonist were added simultaneously (at 0 min), eserine, miotine and prostigmine had no effect. When prostigmine or eserine were allowed to act for 12 min beforehand the tubocurarine effect was halved, but after 12 min exposure to 3393 it was almost abolished.

Combination of 3393 and adrenaline—Bulbring (1946) has shown that when the addition of prostigmine to the isolated diaphragm results in greater contractions, the subsequent addition of adrenaline causes a further augmentation. We have compared the effect of adding 0.25 μ g 3393 together with 50 μ g adrenaline, with the effect of the same dose of 3393 alone. In each comparison

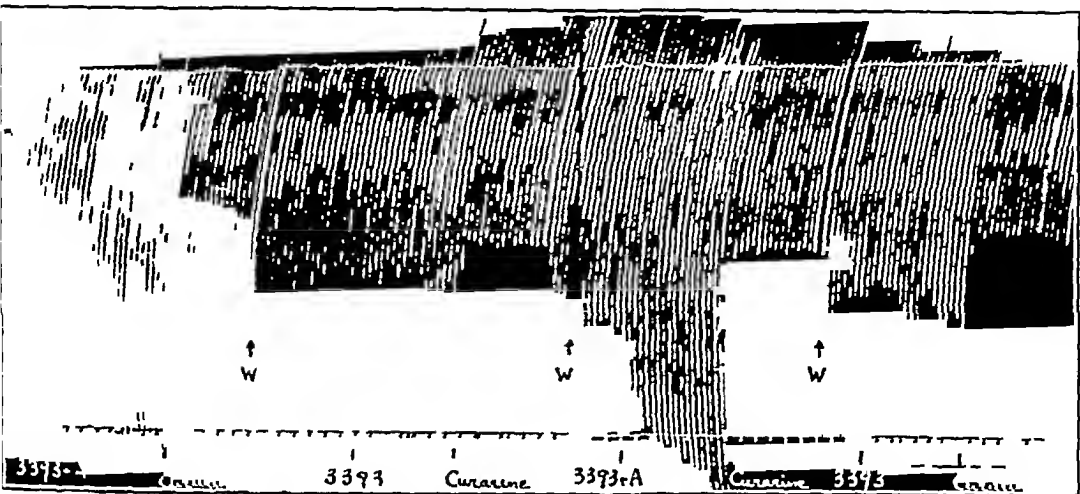


FIG 5—Record as Fig 1. Comparison of anti-curare activity of 3393 alone with that of 3393 combined with adrenaline. Though contractions were greater when 3393 was given together with adrenaline, the final curarine inhibition remained unaltered.

the presence of adrenaline augmented the contractions, as shown in Fig 5. The adrenaline, however, failed to modify the effect of curarine added 6 min later, so that the final result was the same whether adrenaline was present or not.

TABLE III

EFFECT OF GUANIDINE ON MUSCULAR INHIBITION BEFORE AND AFTER TUBOCURARINE (PHRENIC NERVE-DIAPHRAGM PREPARATION OF THE RAT)

Date	Dose of guanidine	Time added before or after tubocurarine	Dose of tubocurarine μ g	Percentage inhibition
Feb 15	Nil	—	80	47
	1 mg.	5 before	80	37
	1 mg.	5 after	80	41
	Nil	—	80	71
Jan. 22	Nil	—	120	37
	1 mg.	with tubocurarine	120	36
	1 mg.	5 before	120	18
	1 mg.	10 before	120	12
	1 mg.	20 before	120	12

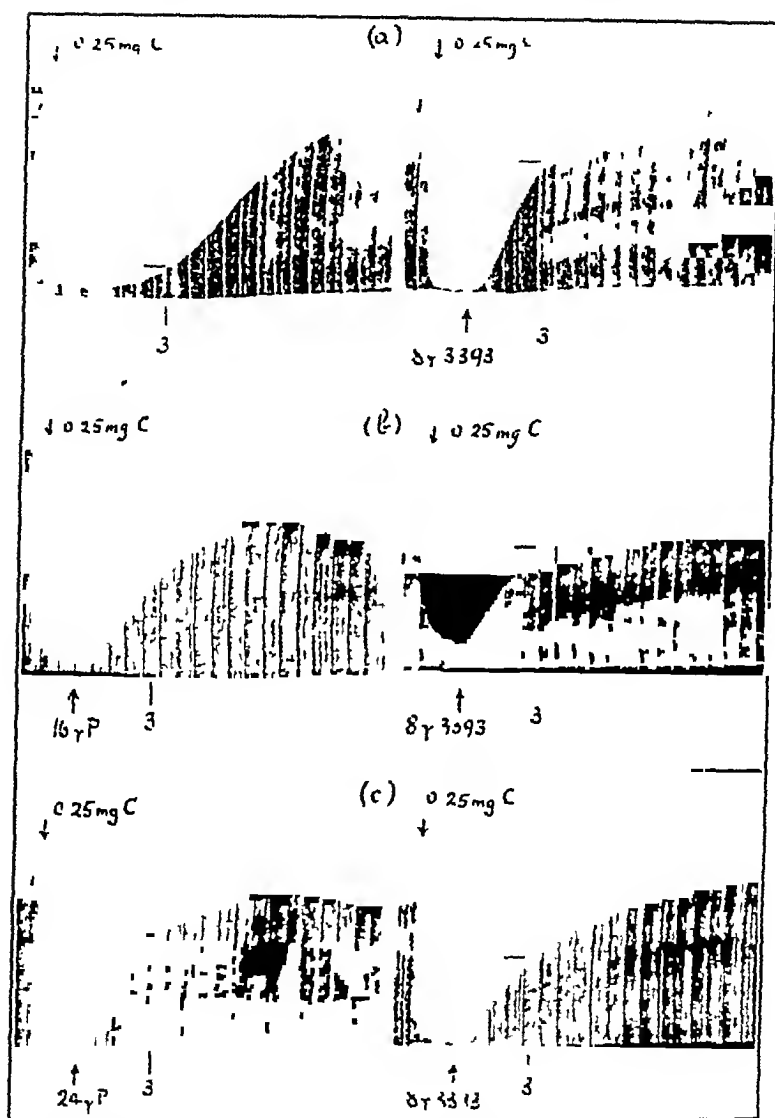


FIG 6—Cat Sciatic gastrocnemius (contractions upwards) Rate of stimulation 12 per min (a) Recovery from the effect of 0.25 mg tubocurarine compared with that after 8 μ g 3393 (b) The same after 16 μ g. prostigmine and 8 μ g 3393 (c) After 24 μ g. prostigmine and 8 μ g 3393

Guanidine and dimethylguanidine—Guanidine is known to exert an action on skeletal muscle, producing fibrillary twitching in low concentration and muscular paralysis in high concentration (Meighan, 1917) It has been used for patients suffering from myasthenia gravis, and some observers record consider-

able improvement (Dodd, Riven and Minot, 1939 1941) The present experiments show that the decurarizing action of guanidine on the rat's nerve-muscle preparation is very weak Concentrations below 1 μg per ml have no action When the concentration is increased to 10 μg per ml a feeble decurarizing activity is shown within a period of 9 min Even at this concentration, the action is uncertain (see Table III) However, a greater effect was obtained by prolonging the time before adding curarine Thus when 1 mg guanidine was added to the bath 10 min before adding 120 μg curarine, the inhibition produced was diminished from 36 to 12 per cent When larger doses of curarine (e.g., 0.4 mg) were used, guanidine had no effect Dimethylguanidine, which also produces fibrillary twitching, was similarly tested Its effect was feebler and less definite than that of guanidine

2 Observations on the cat

Experiments were carried out comparing the anti-curare activity of the different substances in the whole animal For this purpose decerebrate cats were used The left hindleg was prepared so as to record the contraction of the gastrocnemius muscle when the sciatic nerve was stimulated The details of the preparation were as given by Büllbring and Burn (1941), injections were made into the bifurcation of the aorta through a cannula tied into the right external iliac artery and the tension developed in the gastrocnemius was recorded, the resting tension was about 1 kg. The sciatic nerve was stimulated by condenser discharges from a neon-lamp circuit at a constant rate, which was 8 per min in some experiments and 20 per min in others The dose of tubocurarine was chosen to produce a paralysis lasting about 20 min before recovery was complete, this varied in different experiments from 0.25–0.75 mg In testing the action of prostigmine or of its homologues the dose was injected at a precise interval usually 1 min after the injection of the tubocurarine An example of the effect of tubocurarine alone and of tubocurarine followed by 3393 is given in Fig 6 (a) No close comparison between the different substances was found possible by this method but in a series of experiments an approximate relation was established by determining the percentage recovery at a given time (from 3–12 min in different experiments) after the injection of the tubocurarine antagonist Thus in Fig 6 (b) it was shown that 3 min after 16 μg prostigmine the muscle contractions had recovered 38 per cent of their height compared with 56 per cent recovery 3 min after 8 μg . 3393 Therefore 16 μg prostigmine was weaker than 8 μg 3393 Similarly in Fig 6 (c) 24 μg . prostigmine was stronger than 8 μg 3393

The final result obtained from a comparison of the two substances on five cats is illustrated in Table IV In the first experiment it was demonstrated that prostigmine was clearly weaker than 3393 when the two were injected in equal doses In the second experiment it was shown that prostigmine had, however more than one-third of the action of 3393 Finally, in each of three experiments prostigmine was found to have approximately 50 per cent of the activity of 3393 These results agree with those obtained on the rat's diaphragm However, when prostigmine was compared with 3392 in the whole animal no difference was observed between them It should be pointed out that in each experiment several comparisons of the selected doses of the two tubocurarine antagonists were made

TABLE IV

RECOVERY OF MUSCLE CONTRACTIONS IN THE CURARIZED SCIATIC-GASTROCNEMIUS PREPARATION OF THE CAT AFTER PROSTIGMINE AND 3393 (Pr = PROSTIGMINE)

Date	Time of injection	(1) Tubo-curarine mg.	(2) Antagonist and dose (μ g.)	Interval between injections (1) & (2) min	Interval between injection (1) and measurement min	Percentage recovery	Result
12 2 46	2.45	0.30	— —	—	4	5	3393 > Pr
	4.05	"	3393 4	1	4	64	
	4.45	"	Pr 4	1	4	11	
	5.24	"	Pr 20	1	4	58	
	5.54	"	3393 20	1	4	74	
8 3 46	3.10	0.75	3393 4	8	12	68	3393 < 3 \times Pr
	3.41	"	Pr 12	8	12	76	
	4.10	"	3393 4	8	12	36	
	4.42	"	3393 4	8	12	28	
	5.13	"	Pr 12	8	12	90	
29 3 46	12.40	0.25	Pr 16	1	11	10	3393 = 2 \times Pr
	1.07	"	3393 8	1	11	10	
1 4 46	11.58	0.25	— —	—	3	14	3393 = 2 \times Pr
	12.28	"	3393 8	1	3	61	
	12.58	"	Pr 16	1	3	38	
	1.28	"	3393 8	1	3	56	
	2.10	"	Pr 24	1	3	60	
	2.40	"	3393 8	1	3	45	
	3.20	"	Pr 16	1	3	68	
27 3 46					Interval between injection (1) and reappearance of contractions		3393 = 2 \times Pr
	12.29	0.75	3393 4	1	3 min. 40 sec.		
	12.59	"	— —	—	4 " 10 "		
	1.29	"	Pr 8	1	3 " 40 "		
	2.10	"	3393 8	1	3 " 15 "		
	2.46	"	Pr 16	1	3 " 25 "		
	3.24	"	3393 8	1	3 " 15 "		
	3.48	"	3393 16	1	2 " 25 "		
	4.06	"	Pr 32	1	2 " 55 "		

3 Observations on toxicity

It remained to compare the toxicity of these substances in order to discover whether the ratio of the toxic dose to the active dose was greater for 3393 than for prostigmine

White mice were used. Different doses of prostigmine and 3393, calculated per 20 g mouse, were injected into the tail vein. The LD₅₀ of 3393 was 1.2 μ g per 20 g mouse, that of prostigmine was 3.2 μ g per 20 g mouse. When atropine

(40 $\mu\text{g.}$ per 20 g mouse) was given intraperitoneally 1 hour beforehand, the toxicity of both substances was reduced to less than half. For 3393 the LD₅₀ was 2.7 $\mu\text{g.}$ per 20 g mouse, for prostigmine it was 7.9 $\mu\text{g.}$ per 20 g mouse. Thus the ratio without atropine was 2.7 and in the presence of atropine 2.9. As the anti-curare activity of 3393 compares with that of prostigmine as 2:1, and the toxicity as 2.7:1 and in the presence of atropine as 2.9:1, the therapeutic index of 3393 is slightly less favourable than that of prostigmine.

4 The inhibition of cholinesterases by prostigmine and related substances

Prostigmine and similar substances are inhibitors of cholinesterases, but it is still under discussion whether or not their anti-curare effect is fully explained by their affinity to cholinesterase. One of us (Chou) has therefore compared the inhibitory action of prostigmine, miotine, and the two homologues of prostigmine on the enzymic hydrolysis of acetylcholine. Schweitzer, Stedman, and Wright (1939) have compared the inhibitory action of prostigmine and 3393 on the enzymic hydrolysis of butyrylcholine by horse serum; they found a stronger inhibition with 3393. Horse serum, according to Mendel, Mundell, and Rudney (1943), contains chiefly a non-specific cholinesterase, and we have therefore examined preparations from three different sources, viz. (1) human plasma, (2) human red cell haemolysate, and (3) suspensions from the dog's caudate nucleus. Human plasma, like horse serum, contains a non-specific esterase, but red cells and brain suspensions contain a specific cholinesterase.

The rate of hydrolysis was followed manometrically using Warburg's method. The gas mixture was 95 per cent N_2 and 5 per cent CO_2 , and the total reaction volume was made up to 3 ml. The physiological solution employed was Krebs's bicarbonate-Ringer. The experiments were carried out at a temperature of 38°C. In all experiments inhibitor and enzyme were filled into the main compartment of the conical manometric flasks; the reaction was started by tipping the acetylcholine from the side bulb. The amount of carbon dioxide liberated from the 3rd to the 33rd min. after tipping was used for calculating the percentage inhibition. In all experiments the concentration of acetylcholine bromide used as substrate was $6 \times 10^{-3}\text{M}$.

(a) *Experiments on human plasma.*—The hydrolysis of acetylcholine was strongly inhibited by all three members of the prostigmine series (Table V) with

TABLE V

THE PERCENTAGE INHIBITION OF CHOLINESTERASE IN HUMAN PLASMA BY THE PROSTIGMINE SERIES

Inhibitor	Percentage inhibitions at				
	10^{-6}M		10^{-7}M		
Prostigmine	89	90	54	53	40
3392	—	94	—	—	59
3393	—	96	81	—	80

inhibitor concentrations of $10^{-6}M$ and $10^{-7}M$. The percentage inhibitions were in the order Prostigmine < 3392 < 3393

(b) *Experiments on human red cell haemolysate*—The red cell esterases are known to be closely related to the enzyme of nervous tissue. In our experiments the inhibitions were less than with the plasma esterase, this may be explained by the high affinity of the red-cell enzyme to acetylcholine.

Prostigmine and its homologues were examined in a molar concentration of 10^{-6} and the percentage inhibitions were prostigmine, 70, 3392, 82, and 3393

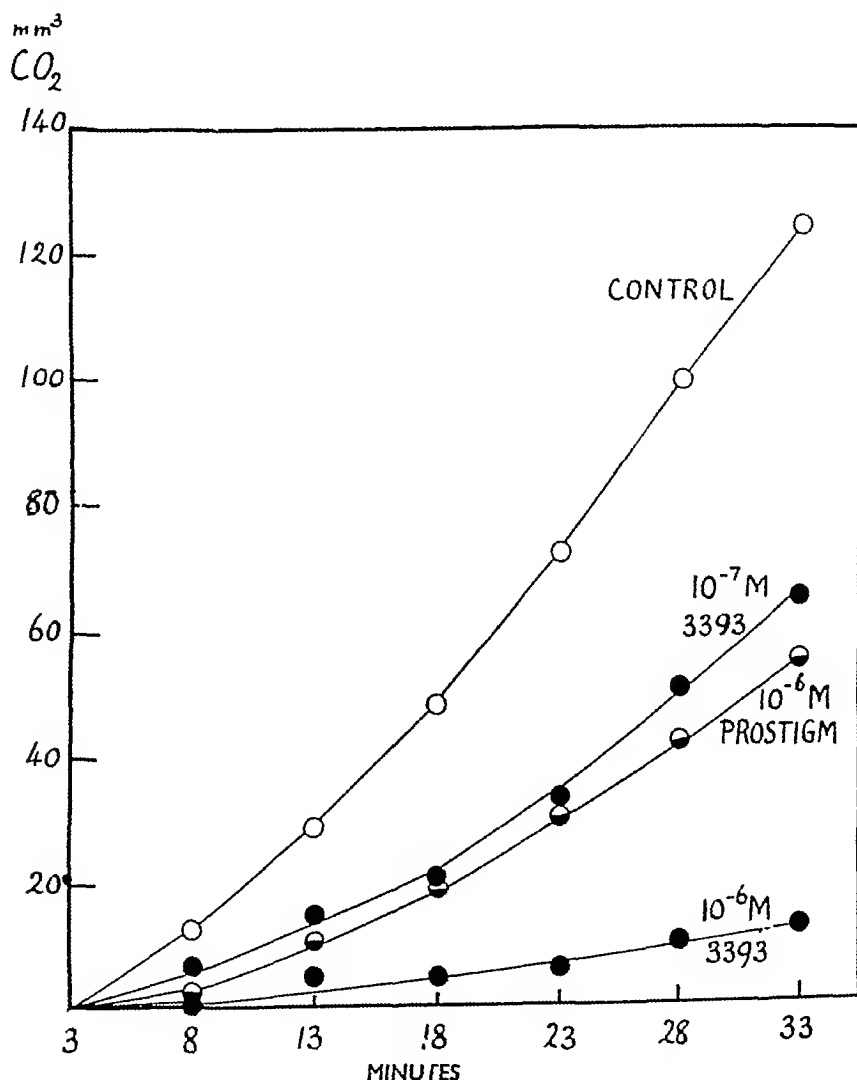


FIG 7—Inhibition of cholinesterase in human red corpuscles haemolysate. Abscissae = time in minutes. Ordinates = mm³ CO₂

94 per cent. Thus, as for the plasma enzyme, the order of inhibitory activity was prostigmine <3392 <3393. In order to obtain a more quantitative estimate of the relative inhibitor properties of prostigmine and 3393, the experiment shown in Fig 7 was carried out. It shows that the inhibition of the red-cell enzyme caused by 10^{-6} M 3393 was only slightly less than that caused by a tenfold molar concentration of prostigmine.

We have also compared the inhibition of the red-cell enzyme by miotine and by prostigmine in two experiments, using the inhibitors at a molar concentration of 10^{-6} . Prostigmine was slightly more effective than miotine.

(c) *Experiments with suspension of dog's brain*—The results obtained with a suspension of the caudate nucleus of the dog confirm earlier observations on the high cholinesterase activity of this tissue. In our experiments 10 mg of brain tissue (fresh weight) were used in each flask. Our results with the three members of the prostigmine series are closely similar to those obtained with human red cells. In 10^{-6} M concentration, the percentage inhibitions were: prostigmine, 60; 3392, 78; and 3393, 91 per cent.

DISCUSSION

It is not known whether the anti-curare activity of substances which inhibit cholinesterase is solely due to this property or whether another mechanism is involved. In the experiments described in this paper it was found that substitution in the prostigmine molecule of one or two of the methyl groups attached to the N atom of the basic radical by ethyl groups led to an increase in anti-curare activity; similarly, this change led to an increase in anti-cholinesterase activity. Thus the order of potency was found to be prostigmine <3392 <3393. But while the substitution of two methyl groups in the prostigmine molecule by two ethyl groups doubled the anti-curare activity, it caused a greater increase in the inhibition of cholinesterase. When miotine, which does not resemble prostigmine so closely, was tested, it was found to have the same anti-curare activity as prostigmine, but to be definitely weaker in inhibiting cholinesterase. In the prostigmine homologues the change in anti-curare action and anti-cholinesterase action is in the same direction, though not parallel. It remains doubtful whether a general parallelism exists between the two properties for substances other than those of closely related chemical structure.

SUMMARY

1 The activity of prostigmine homologues, eserine and miotine as antagonists to tubocurarine was estimated on an isolated muscle preparation and in the whole animal.

2 The isolated phrenic nerve-diaphragm preparation of the rat was found to be a quick method for comparing large numbers of compounds. The results

obtained with this method were in good agreement with those obtained *in vivo* on the cat's sciatic gastrocnemius

3 The activity of prostigmine homologues as inhibitors of cholinesterase increases in the same direction as their anti-curare activity. It is doubtful whether a similar parallelism holds for molecules of different structure.

We wish to express our thanks to Dr H Blaschko for his great help and advice with the manometric experiments.

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EFFECT OF STREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS

BY

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There have been several favourable reports from U.S.A. on the efficacy of streptomycin as a chemotherapeutic agent, with particular reference to tuberculosis (e.g., Feldman and Hinshaw, 1944, Feldman, Hinshaw, and Mann, 1945, Youmans and McCarter, 1945)

The present paper reports investigations carried out during the past year with a strain of *Mycobacterium tuberculosis* (human), the results of which confirm the observation that streptomycin exerts a marked suppressive action on guinea-pig tuberculosis

EXPERIMENTAL

Strain—A virulent strain of *M. tuberculosis* (human), H.418 supplied originally by Dr Ungar of Messrs Glaxo, Ltd, was employed for all tests. When a three-week growth from a Löwenstein slope was used, 0.0001 mg. of this strain consistently gave rise to a progressive and fatal disease within 6 months.

Animals—Guinea-pigs from the laboratory-bred stock and weighing approximately 500 g. were used. All animals received an ample diet, including green vegetables daily, and cod liver oil twice weekly.

Streptomycin—For the supplies of streptomycin hydrochloride I am indebted to Mr M. Lumb, M.Sc., of this Division, who prepared the culture filtrates, and to Drs Short, Peak, Coppock, and Falconer, who supervised the extraction and preparation of the solid. The material used was, in general, of a potency ranging from 89 to 450 u./mg. For injection the solid material was dissolved in sterile saline or Ringer's solution.

In vitro Tests

When the floating pellicle test with Douglas's modification of Long's medium was used strain H.418 was inhibited completely at 10 u./ml. and there was some inhibition at 1 u./ml. When Youmans's (1944) submerged culture test and the above medium, with 10 per cent ox serum added, were used, the effects varied with the size of the inoculum, as has been noted with other substances. Table I gives typical results of this test, and it will be seen that 6.5 u./ml. inhibited the growth of a small inoculum, of which the control grew well whilst quite large amounts of streptomycin had little or no effect on much larger inocula.

Tuberculostatic action of the blood of treated guinea-pigs

It was readily shown by the Brownlee test (1945) that the heart blood of treated guinea-pigs inhibited the growth of tubercle bacilli. For this test the blood was mixed with the modified Long's agar medium, and sowed with strain H.418. The blood taken up to three hours after the subcutaneous injection of 1,500 units of streptomycin showed this inhibition of growth of the organisms.

TABLE I

SUBMERGED GROWTH OF *M. TUBERCULOSIS* IN PRESENCE OF STREPTOMYCIN (2 WEEKS)

Concentration of streptomycin	Inoculum size per 10 ml					
	0.5 mg.		0.05 mg		0.005 mg.	
Nil (control)	+++	++	++	++	++	++
6.5 u/ml	+++	+++	++	++	-	-
65 u/ml	+++	+++	+	+	-	-
650 u/ml	+++	+++	++	+	-	-

In vivo Tests

Test I—In a preliminary test a daily dosage of 2,000 u/pig/day was given to six guinea-pigs for 8 weeks 500 units being given subcutaneously four times daily, at 3 hourly intervals, commencing 4 days after the intraperitoneal injection of 0.0005 mg strain H 418. None of the six controls or the six treated animals died during the 8 weeks of the experiment, but on necropsy the results were considered promising, especially in view of the relatively small dosage. All the guinea pigs showed macroscopic evidence of the disease: the control animals had very extensive tuberculosis of the spleen, liver, and lungs, and a large lesion at the site of injection; in contrast, none of the treated guinea pigs had a local lesion and only one had more than scattered tubercles in the spleen and lungs. Spleen cultures of all animals, both treated and controls, were positive.

Test II—In view of the promising results of Test I and the availability of more streptomycin a second test was made, using a much larger dose of the drug—i.e., 10,000 u/day/pig. All animals received an intramuscular infecting dose of 0.0001 mg of strain H 418. Thirty six guinea pigs were divided into three groups:

Group I—12 controls

Group II—12 treated for 14 weeks from date of infection

Group III—12 treated for 11 weeks, starting 24 days after infection (when positive with 0.05 ml of 1/100 tuberculin)

Groups II and III received four subcutaneous doses of 2,500 units streptomycin every day at 8.30 a.m., 11.30 a.m., 2.30 p.m., and 5.30 p.m. The animals were weighed fortnightly until the first part of the experiment was terminated after 14 weeks. At this stage nine animals of each group were killed, the remaining three being left without further treatment in order to ascertain the consequences of discontinuing administration of the drug.

Before being killed each animal was again tuberculin tested. After necropsy cultures were made from the spleens and portions were injected into healthy guinea-pigs. Sections of spleen, lung, and liver were preserved for histological examination.

An approximate numerical assessment of the macroscopic evidence of disease was carried out by the method of Sher and Kloeck (1946), each organ having the following maximum value:

Lymph nodes	8	Liver	28
Spleen	24	Lungs	40

RESULTS

As can be seen from Table II, the results were encouraging. Six of the nine guinea-pigs treated from the date of infection and two of the eight which did not receive treatment until 24 days after infection showed no macroscopic evidence

of tuberculosis, the other treated animals showed only slight glandular involvement. All the control animals showed widespread macroscopic tuberculosis which would obviously have been fatal in a few weeks spleen, glands, liver, and lungs were involved and the group assessment was 33 (average of the 9 pigs), against 0.6 for Group II and 2.0 for Group III. Before the animals were killed, some of the tuberculin tests on the treated guinea-pigs were doubtful, whereas all those made on control animals were strongly positive.

TABLE II
THE INFLUENCE OF STREPTOMYCIN IN GUINEA-PIG TUBERCULOSIS

Group	Treatment	Length of infection	Deaths	T B assessment	
				Individual animals	Group average
I Controls					
9	Nil	14 weeks	0/9	12, 22, 38, 55, 33, 39, 37, 45, 16	33
3	Nil	22 weeks	1/3	58, 78, 85	74
II Treated from date of infection					
9	10,000 u./day for 14 weeks	14 weeks	0/9	0 0 0 0 2, 2, 2, 0, 0	0.6
3	10,000 u./day for 14 weeks then nil for 8 weeks	22 weeks	0/3	24, 8, 26	19
III Treated when tuberculin positive (24 days after infection)					
9	10,000 u./day for 11 weeks	14 weeks	*1/9	2, 2, 0 0, 2, 4, 4, 4	2.2
3	10,000 u./day for 11 weeks, then nil for 8 weeks	22 weeks	0/3	49, 49, 39	44

* This death (premature) was not due to T B

Spleen cultures and injection of spleens into normal guinea-pigs—All spleens were cultured on Lowenstein medium. Six out of nine of the control samples gave a positive response, whilst of the three negative ones two were macroscopically infected. In both Group II and Group III two spleens out of nine gave a positive result.

Half of each spleen of eleven of the treated guinea-pigs (six from Group II, five from Group III) was ground with sand and Ringer's solution, and the supernatant fluid from each preparation was injected into a normal animal. The two controls showing the least degree of infection were also tested in this way. In all cases, except one of Group III, the guinea-pigs developed widespread tuberculosis in two months.

Development of tuberculosis on cessation of streptomycin treatment—Three guinea-pigs from each group were left on test after cessation of streptomycin treatment in order to ascertain whether the disease would flare up. After two months, one control had died, and all other pigs were tuberculin positive and showed at necropsy macroscopic tuberculosis of spleen, lungs, and glands. As would be expected, the disease was far more extensive in the controls than in the treated pigs, but even the latter showed marked glandular and spleen involvement. Assessments are given in Table II.

Toxicity—At the beginning of the experiment streptomycin made the animals' skins very sensitive and there was considerable irritation. This effect was not observed after several weeks, and may have been due to the impurities present in the early batches used. At necropsy there were no signs of any toxic effect and all pigs appeared healthy throughout the experiment and were gaining in weight.

DISCUSSION

The results, although of necessity limited to small numbers of animals owing to the small amounts of streptomycin available, confirm Feldman's work (1944, 1945) regarding the marked suppressive effect of the drug on experimental tuberculosis of guinea-pigs. Under our experimental conditions, 10,000 u/day were effective, although the results of spleen culture and the flare-up tests showed that virulent and viable bacilli were still present after 14 weeks' treatment. During treatment the disease did not progress beyond minor involvement of glands and spleen, and in several pigs it was macroscopically absent. The presumption is that if the experiment had been continued longer these pigs would not have developed the disease so long as treatment was maintained, whereas the controls were obviously succumbing to generalized tuberculosis. Feldman's failure to eliminate tubercle bacilli from the body has also been confirmed by these experiments. It is obviously important to discover whether or not more extensive treatment will cure the disease entirely. This and other related studies are now in hand in these laboratories.

SUMMARY

- 1 The marked suppressive effect of streptomycin on experimental tuberculosis of guinea-pigs has been confirmed.
- 2 Under our conditions the disease was not entirely eliminated from the treated animals.

The author wishes to thank Sir Jack Drummond, F.R.S., and Mr C. E. Coulthard for their interest in this work, and Miss B. Bailey, B.Sc., for her assistance in the *in vivo* work.

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THE RELATIONSHIP BETWEEN SURVIVAL TIME AND DOSAGE WITH CERTAIN TOXIC AGENTS

BY

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In toxicity experiments with chemical warfare agents it was frequently noticed that animals which had received the largest doses died more quickly than those receiving smaller doses. The investigations described here were carried out in order to find whether a quantitative relationship existed between dosage and survival time and, if so, how best this relationship could be expressed and used. It has not been possible for the present authors to pursue this investigation very far, but in view of the interesting results so far obtained it is hoped that others may extend this study to other substances.

The substances used in our investigations were mustard gas, $S(CH_2CH_2Cl)_2$, and phosgene gas, $COCl_2$. When breathed by animals these substances exert their effect in quite different ways. Animals dying after exposure to phosgene consistently showed, at autopsy, a picture of pulmonary oedema, those dying after exposure to mustard gas vapour, however, presented a more varied pathology, pseudo-membranous tracheitis, pulmonary oedema, broncho-pneumonia, and enteritis being the predominant autopsy findings.

The survival times of the animals were quite different. With mustard gas animals may die from the direct effect of the vapour 20 days after exposure, whereas with phosgene nearly all animals die in the first 48 hours.

Mice exposed to mustard gas

Mice were exposed to mustard gas vapour in a constant-flow apparatus. The advantage of this type of apparatus over the static chamber is that the concentration of gas does not tend to fall off during the exposure as it does with the latter owing to adsorption on to the surface of the chamber and the animals' fur. In the constant-flow apparatus a continuous flow of air-gas mixture of the required concentration is drawn through the chamber. In the experiments described here the volume of the chamber was 20 litres and the flow of air-gas mixture was 200 litres per minute. The liquid mustard was evaporated into the air stream at the required rate by means of an electric heating coil. The atmosphere in the chamber was continuously sampled throughout the exposure and accurate determinations (within about 1 or 2%) of the concentrations in the chamber could be made.

240 male albino mice (of weight 25–30 g.) were mixed up together and 8 groups of 30 mice selected at random. The groups were exposed all on the same day to 8 different

concentrations of mustard vapour. The exposure time in each case was 10 minutes. The exposures were carried out in wire cages which had separate compartments for each mouse in order to avoid the possibility of the mice huddling together and breathing through each other's fur.

The mortalities at the end of successive 24-hour periods are shown in Table I.

TABLE 1

DISTRIBUTION OF SURVIVAL TIMES IN GROUPS OF 30 MICE EXPOSED TO VARIOUS CONCENTRATIONS OF MUSTARD VAPOUR FOR 10 MIN IN A CONSTANT-FLOW APPARATUS

Concentration in mg./cu m	Time in days																										Total mortality
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
A 67									2		2			2		1				1						8/30	
B 93							5		1		2			3	2				1						1	15/30	
C 116						1	1		1	1	1	4		3			2	2	1				1			18/30	
D 160				1	2	2	2	5		2	2		1		1			1								17/30	
E 260			4	2	2	2	2	6	7		1	3							2		1					30/30	
F 300				5	8	2	4	2	3		2						1	3								30/30	
G 375			5	9	7	4	3	2																		30/30	
H 505	3	11	10	6																						30/30	

Analysis of results

(i) As a preliminary analysis the median time for each group was plotted against the dosage, in general, it was clear that median survival time decreased with increase of dosage, but not as a linear function, in fact, a smooth curve drawn through the points resembled a rectangular hyperbola. A second very noticeable feature of the data was the increase in spread of the observations in the groups with larger average survival times.

(ii) The original data were converted to logarithms and, in the groups where all the animals died, the means and standard deviations were computed in the usual way. In the groups in which some animals survived, estimates of the means and standard deviations were obtained by plotting the logarithms of the individual observations in each group on a probit scale and estimating the mean from the intersections of the best "eye-fitted" line with the ordinate at 5 probits, and the standard deviation from the reciprocal of the slope of the line (Bliss, 1936, and Gaddum, 1945). It seemed doubtful whether very accurate estimates could be obtained by this method, since there appeared to be a general tendency for the lines to be convex upwards (i.e., for the distributions to be positively skewed), and the assumption of normal distribution, on which this method is based, therefore seemed to be invalid. A second very noticeable feature about the data was that in spite of the log transformation the means and standard deviations for the groups were still highly correlated (the product moment correlation coefficient for the unweighted data was about 0.93, which is highly significant $P < 0.001$).

When these estimates of the mean log survival times were plotted against the logarithms of the dosage there appeared to be a linear relationship. A straight line drawn through the points had a slope of about -1 , suggesting a reciprocal relationship between time and dosage.

(ii) The original data were therefore converted to reciprocals, and means and standard deviations of the reciprocal survival times for the groups were found as before. It was noticeable that when the results for each group were plotted on a probability scale there seemed to be no tendency for the points to be curvilinear, which suggested that after this transformation the data were more nearly normally distributed. A second feature of the use of this transformation was the apparent stability of the variance. (The product moment correlation coefficient for the unweighted data was about -0.26 , which is quite non-significant $P=0.5$).

The values for the estimates of means and standard deviations for the groups using the logarithmic and the reciprocal transformations are given in Table II.

TABLE II
VALUES FOR THE ESTIMATED MEANS AND STANDARD DEVIATIONS OF TRANSFORMED SURVIVAL TIMES (DAYS)

Group	Mustard dosage (Ci) mg min/cu m	Log transformation (log days)		Reciprocal transformation (100 \ days ⁻¹)	
		Mean	Standard deviations	Mean	Standard deviations
A	670	1.43	0.31	1.3	6.8
B	930	1.30	0.35	3.9	7.8
C	1160	1.27	0.25	5.2	4.3
D	1600	1.10	0.21	7.1	6.0
E	2600	0.88	0.19	14.6	7.0
F	3000	0.87	0.20	14.6	5.4
G	3750	0.71	0.12	20.1	5.1
H	5050	0.60	0.10	26.0	6.4
		Correlation coeff between mean and S.D. = -0.93 $P=0.001$		Correlation coeff between mean and S.D. = -0.26 $P=0.5$	

When the estimates of the mean reciprocal survival times were plotted against the dosage the points appeared to fall near a straight line.

It is clear that the estimates of mean reciprocal survival time were not of equal reliability, since some were estimated from complete data in the ordinary way, whilst others had to be obtained by graphical methods. Some system of weighting should therefore be used to allow for this. Bliss (1936) and Stevens (1936) have worked out a method for an analogous case in which by successive approximation the maximum likelihood estimates of the means and standard deviations of the truncated normal curve can be calculated to any required degree of accuracy. They also give tables of a factor E which enables one to

calculate the variance of the mean of the truncated curve. This factor E may be used to furnish a weighting factor N/E .

When the data are complete (i.e., when all the animals react), E is equal to unity and the weighting factor becomes equal to N , the number of animals in the group. When the exposure curve is truncated owing to some animals failing to react, N/E will give the equivalent number of animals which would have given as accurate a result if they had all reacted. With the reciprocal transformation, all the points seemed to fit the dosage-probit line and there was no difficulty in deciding at what point truncation* had occurred, it was in every case determined simply by the proportions of animals dying.

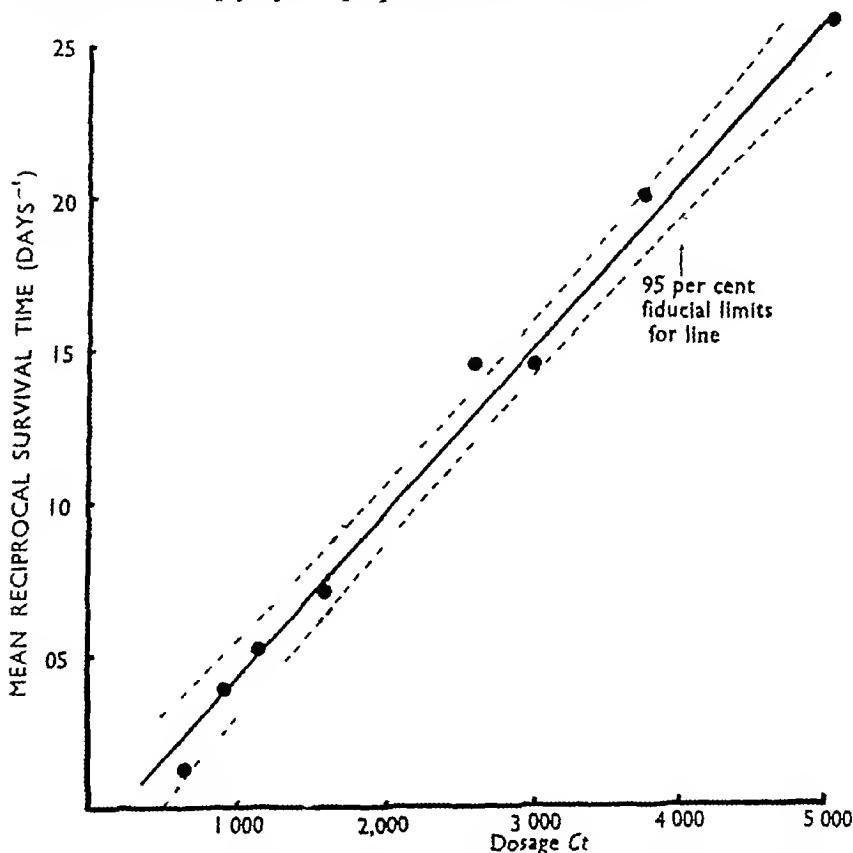


FIG 1—*Mustard*. The mean reciprocal survival time for groups of mice exposed to various dosages of mustard gas vapour, showing the line of best fit with its 95 per cent fiducial limits

*Bliss (1936) dealt with a similar problem (reaction time of organisms when immersed in a toxic solution or gas). His data usually showed a tendency for the points corresponding to the last few organisms to react to fall away from the line. He regarded these organisms as atypical and found the point of truncation by noting where the line appeared to change in slope.

To test the hypothesis that the reciprocal of the survival time was linearly related to the dosage a regression line was fitted between the means of the reciprocal survival times and the dosages. This line, together with the approximate 95 per cent fiducial limits, is shown in Fig 1.

An alternative hypothesis is that the survival time is proportional to the dosage (i.e., that the line passes through the origin), a second calculation was performed, therefore, and the best-fitting line obtained with the restriction that it should pass through the origin. The "goodness of fit" of the two lines may now be tested, since we may compare the sum of weighted squares of residuals in each case with the pooled "within groups" variance, the ratio of these two quantities will be distributed approximately as χ^2 (since the numbers of observations involved in the between groups variance is large). We obtain

TABLE III

<i>Hypothesis (1)–</i>			<i>Hypothesis (2)</i>		
DOSE AND RECIPROCAL OF SURVIVAL TIME LINEARLY RELATED			DOSE AND RECIPROCAL OF SURVIVAL TIME PROPORTIONAL		
χ	Degrees of freedom	P (approx.)	χ^2	Degrees of freedom	P (approx.)
3.43	6	0.75	5.03	7	0.5

It is clear that either of the above hypotheses is acceptable.

A distinction is here drawn between the two possibilities, since it seems likely that for some toxic substances a definite "threshold" effect may exist when, although the relationship might be linear, the line would not pass through the origin. It seems useful to keep the more general case in mind, even though in our work we have found no significant departure from proportionality.

TABLE IV

DISTRIBUTION OF SURVIVAL TIMES IN GROUPS OF RATS EXPOSED TO VARIOUS CONCENTRATIONS OF PHOSGENE GAS FOR 10 MIN. IN A CONSTANT-FLOW APPARATUS

Concentration in mg./cu m.		Time in hours																								Total mortality	
		3	3½	3¾	3¾	4	4½	4¾	4¾	5	5½	6	6½	7	8	9	10	11	12	14	16	20	24	30	40		60
T	151																1			1						1	7/12
U	156														1				2	1						2	14/20
V	300									1			1						1	1			1		4		6/12
W	411										1			1	2					1		1					10/12
X	589									2	1	1	1		2	2		1	2								12/12
Y	702									1	1	2	2		1	2				1				1			12/12
Z	923	1		1	2	3	1			1	4	1	1	1	2	2			1								20/20

Rats exposed to phosgene gas

The apparatus used for the phosgene exposure is described fully elsewhere (Box and Cullumbine, 1947). A number of groups of albino rats (weight 115–125 g.) were exposed all on the same day to various concentrations of phosgene, as with mice, each rat was exposed in a separate compartment. The exposure time was 10 minutes in each case. The rats were inspected at the times shown and the mortalities recorded. The results are given in Table IV.

Analysis of results

The results were analysed exactly as before, the features of the distribution of survival time being remarkably similar. Again we found a general decrease in survival time with increase in dosage, which assumed a linear form when plotted on a log-log scale with slope approximately equal to -1 . Again the spread of observations was correlated with the mean value and this was still the case on transforming to logarithms ($r=0.87$, $P=0.01$), but not the case with reciprocal transformation ($r=-0.28$, $P=0.5$). As before, plots of probit mortality against log dose suggested positively skew distribution with the log transformation, but normal distribution with the reciprocal transformation.

The values for the estimates of means and standard deviations for the individual groups are given in Table V.

TABLE V

VALUES FOR THE ESTIMATED MEANS AND STANDARD DEVIATIONS OF TRANSFORMED SURVIVAL TIMES (HOURS)

Group	Phosgene dosage (Cr) mg. min/cu m	Log transformation (log hours)		Reciprocal transformation (100 \times hours ⁻¹)	
		Mean	Standard deviations	Mean	Standard deviations
T	1510	1.45	0.34	2.8	5.0
U	1560	1.43	0.33	3.3	5.2
V	3000	1.34	0.47	4.0	10.9
W	4110	1.07	0.27	8.9	6.0
X	5890	0.94	0.24	13.0	5.7
Y	7020	0.80	0.14	16.7	5.8
Z	9230	0.72	0.13	20.1	5.7
		Correlation coeff between mean and S.D. = 0.87 P=0.01		Correlation coeff between mean and S.D. = -0.28 P=0.5	

Regression lines were calculated exactly as before and the line obtained on the hypothesis of simple linearity is shown in Fig 2.

Again the alternative hypothesis of proportionality between mean reciprocal time and dosage was tested, and the values obtained for χ^2 in the test of goodness of fit are given in Table VI.

TABLE VI

<i>Hypothesis (1)</i> DOSE AND RECIPROCAL OF SURVIVAL TIME LINEARLY RELATED			<i>Hypothesis (2)</i> DOSE AND RECIPROCAL OF SURVIVAL TIME PROPORTIONAL		
χ^2	Degrees of freedom	P (approx)	χ^2	Degrees of freedom	P (approx)
1.81	5	0.9	2.15	6	0.9

As before, neither of these tests demonstrates significant departure from the respective hypotheses

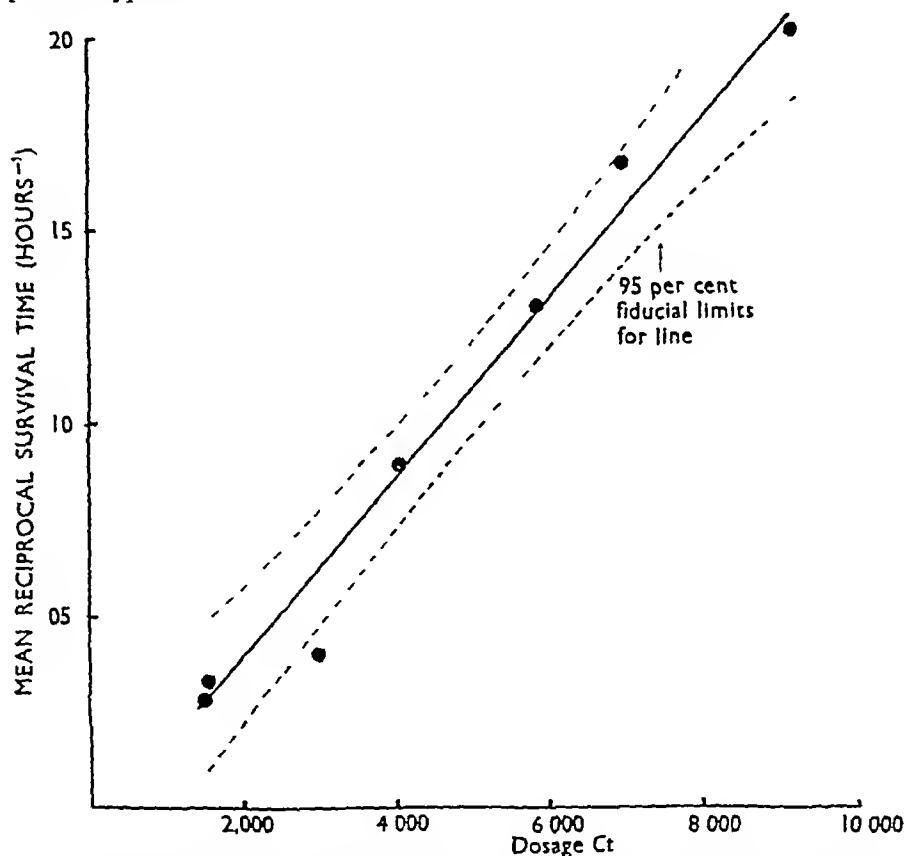


FIG 2—*Phosgene* The mean reciprocal survival time for groups of rats exposed to various dosages of phosgene gas, showing the line of best fit with its 95 per cent fiducial limits.

DISCUSSION

Interpretation and use of the relationship

The use of survival time in pharmacological experiments has been largely confined to the standardizing of cortical extracts (Bulbring, 1937, *et al*) We

believe that where substantial correlation can be shown between dosage and a function of survival time, its use may be considerably extended. With the two substances and species of animals investigated our experiments suggest (i) Rate of dying (i.e., the reciprocal of the survival time) is directly proportional to the dosage, (ii) rate of dying tends to be homoscedastic (i.e., the variance is roughly constant for different survival times), (iii) rate of dying is probably more nearly normally distributed than survival time or log survival time. These facts were used in the design and analysis of experiments, including many to assess the value of suggested therapies for chemical warfare agents. In this type of experiment it is a great advantage to be able to test a large number of factors at once in accordance with the principles of experimental design described by Professor R. A. Fisher and his followers. For example, the injection of a certain substance may be suggested as a possible treatment for a certain type of poisoning, but normally there will be many uncertainties, such as the correct dose, the best route of administration, whether it should be given in single or divided doses, and so on. We could, of course, try to guess what would be the most promising combination of these factors, expose a control and treated group to (say) an LD₉₀ of the poison, and observe whether any significant reduction of mortality in the treated group occurred. If it did, then attempts to enhance it by varying other factors could be carried out later. The disadvantages of this type of experimentation are (i) if we guess wrongly we may miss the treatment altogether, (ii) it is very uneconomical in the use of animals and (iii) it cannot detect interaction between factors.

By means of a continuous variate, such as transformed survival time, many of the factors can be introduced into the first experiment *without increasing its size or losing efficiency in testing significance* (Fisher, 1942). Difficulties occur, however, when attempts are made to use factorial schemes in which percentage mortality is the variate. The most important of these is the lack of range of this type of experiment. Usually we try to get our stock of animals as uniform as possible, but the more uniform we make them the smaller will be the range over which we shall get a response between 0 and 100 per cent mortality. When a number of factors are introduced to be tested simultaneously, the range of the effects will usually be increased, this will often result in a number of groups with 0 or 100 per cent mortality which have little weight and cannot be compared one with the other. We can widen the range of the experiment by carrying it out at more than one dosage level, but this type of experiment will almost certainly have a low overall efficiency owing to the number of groups with little weight. Further, if we try to introduce a number of factors and dosage levels, we shall soon have to increase the overall size of the experiment, otherwise the individual group will become too small. In this way when a quantal response is used the advantage of the factorial design tends to be lost. Provided that the groups are large enough, this type of experiment can be analysed by the use of the transformation $x = \sin^{-1} \sqrt{p}$ (where p is the proportion dying)

This transformation overcomes the difficulty of inequality of variance in the groups, its disadvantage, however, is that the transformed variate will not be a linear function of dosage. However, if we use a transformation which is a linear function of dosage (i.e., the probit transformation), the weights will depend upon the expected values and be different for different groups. Finney (1943) has given the solution in this case, but his analysis is by multiple regression and becomes laborious when there are many factors, especially if some of the interaction effects have to be included in the analysis.

In view of difficulties of this kind arising out of the use of a quantal response (percentage mortality), wherever it could be shown that some function of survival time was closely correlated with dosage, factorial experiments have been carried out with this function as the variate. The procedure was to give very large doses (usually 3–5 times the LD50), which it was known would probably result in 100 per cent mortality even if the treatments were fairly effective, the therapies were then judged by the increase in survival time. For example, with mustard gas and phosgene, the use of the reciprocal transformation allows the ordinary methods of analysis of variance to be correctly applied. As an additional safeguard the most promising of the treatments may be compared by using percentage mortality and (if the experiment is carried out at more than one dosage level) by calculating the index $I_{LD50} = \frac{\text{LD50 for treated group}}{\text{LD50 for control group}}$, this

index will give a true measure of the effectiveness of the treatment and is a similar measure to M (the log of the ratio of the potencies) used by Gaddum (1933). It is interesting to note that I is a function of the percentage mortality and b (the slope of the probit-dose line), so that unless b is known a test at one dosage level using percentage mortality as the variate does not provide us with any information about the *absolute effectiveness* of the treatment, e.g., it is possible for two equally good therapies to give quite different reductions in mortality if the values of b in the two tests are different. However, in the case of survival time, the ratios of the means of the transformed survival times in control and treated groups will give a measure of effectiveness which is independent of the standard deviations of the groups. For instance, we found that, where the reciprocal transformation is appropriate, $I_T = \frac{\text{Median survival time of treated group}}{\text{Median survival time of control group}}$ gives a measure of effectiveness, which is often a good approximation to I_{LD50} .

Even if a relationship can be established between transformed survival time and dosage for a particular case, it will *not* follow that the method outlined here will necessarily be appropriate for the testing of therapies. Examples may occur where a therapy will increase the survival time without reducing mortality. Usually, however, a knowledge of the probable mechanism of a therapy will enable one to judge whether this method of experimentation will be appropriate. In the opinion of the authors the technique can often be used profitably, side

by side with more orthodox methods, and they have conducted many experiments on these lines, some of which appear elsewhere (Cullumbine and Box, 1946, Box and Cullumbine, 1947)

Analysis of experiments in which some groups are incomplete

Occasionally when using factorial experiments of the type outlined above, a group or groups will partly, or wholly, survive. When all or most of the animals survive in a group or groups in marked contrast to other groups, usually no analysis will be needed to establish the superiority of the corresponding treatments. Experiments in which there are a few survivors in one or two groups only are not so easily dealt with.

(i) Maximum likelihood estimates of the means in the incomplete groups may be obtained by the method of successive approximation given by Bliss (1936) and Stevens (1936). The solution will then be given by regression analysis using the appropriate weighting coefficients, however, the labour involved in the calculations is scarcely justified.

(ii) Provided at least 50 per cent have died in all groups, the medians of the transformed values may be used and the ordinary analysis of variance procedure adopted. A certain amount of information is lost when this method is used, but with small groups the loss is not large. Pearson and Adyanthaya (1928) give the following values

Sample size using median	2	3	4	5	7	10
Equivalent sample size using mean	2	3	4	4	5	8

(iii) The means may be estimated in the incomplete groups by graphic analysis and the experiments analysed as though the ordinary analysis of variance technique were appropriate (i.e., as though the means of the groups were all known with equal accuracy). This method is probably sufficiently accurate provided that the number of animals failing to respond is not too great.

(iv) Where the reciprocal transformation is appropriate, the "rate of dying" of an animal which survives is $\frac{1}{\infty}=0$, so that with this transformation we could score these animals as zero and carry out the analysis in the ordinary way. In our opinion this approximation will be more inaccurate than (iii). If the "within groups" variance is used in the analysis the truncated groups should be omitted from its calculation, since the values will be too small. In general it is better to use high order interactions between group means as the "error" estimate. We find that (iii) gives a satisfactory approximation and utilizes all the information without complicating the analysis.

SUMMARY

1 Experiments are described in which mice were exposed to mustard vapour and rats to phosgene gas. The properties of the survival time, its logarithm and reciprocal were investigated. Using the reciprocal transformation, the transformed

variate (rate of dying) was proportional to the dosage, had stable variance, and appeared to be more nearly normally distributed than time or log time

2 The use in appropriate circumstances of relationships of this nature is discussed and the advantages of factorial experiments using transformed survival time are indicated

3 Methods are suggested for dealing with the data when in some groups the animals do not all die

We should like to express our indebtedness to Prof R. A. Fisher for discussing this problem with one of us and for suggesting the use of the reciprocal transformation

Our thanks are due to the Director-General of Scientific Research (Defence) for permission to publish these results, and to our colleagues at Porton for their advice and criticism

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THE EFFECT OF EXPOSURE TO SUB-LETHAL DOSES OF PHOSGENE ON THE SUBSEQUENT $L(Ct)_{50}$ FOR RATS AND MICE

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It has been suggested that when dogs and goats survive doses of phosgene gas (COCl_2) their subsequent susceptibility is apparently lessened. The effect was explained as being probably due to selection, since the more susceptible animals were killed, the remainder would be more resistant and their average susceptibility lower. The experiments described in this report were carried out in order to obtain further information about the matter.

EXPERIMENTAL

Preliminary experiments were carried out to ascertain the highest concentration to which rats and mice could be exposed for 10 min without causing death. It was found that exposure of rats and mice to dosages (Ct)* of 800 and 600 mg.min/cu.m ($t=10$ min) respectively did not normally produce any deaths although the animals showed all the symptoms of severe phosgene poisoning. These dosages were therefore used throughout the work as the preliminary or "pregassing" doses.

The exposure to phosgene was carried out in a small chamber (20 litres capacity) in a constant flow of 200 litres/min of an air phosgene mixture of the required concentration. The apparatus is shown in Fig 1. The atmosphere in the chamber was sampled at 10 l res/min throughout the whole of the exposure.

*The dosage to which animals were exposed is measured here by multiplying the mean concentration (C) measured in mg/cu.m by the time of exposure t (Hence Ct in mg.min/cu.m). The time of exposure (t) was 10 min in all experiments unless otherwise stated.

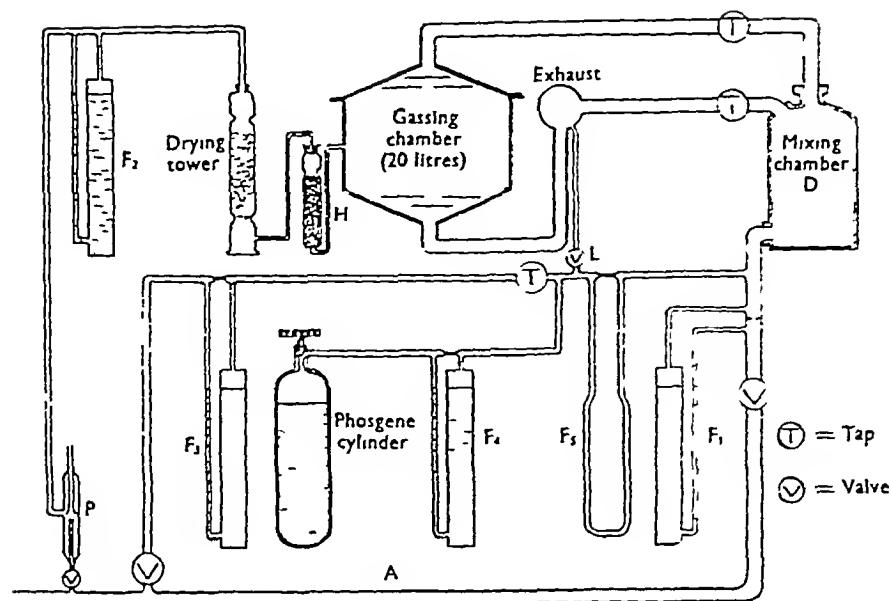


FIG 1—The constant-flow phosgene apparatus. 200 litres/min. of air was blown along the main air line A and measured by flowmeter F_1 . Phosgene (measured by F_2) was mixed with diluting air (measured by F_3). The mixture regulated by valve L was measured by F_4 and mixed in chamber D with the main air stream. The diluted air-phosgene mixture could then be made to pass through the chamber or run to waste as required. A suction pump (P) drew a continuous sample at 10 litres/min. through the sampling bubbler H.

The effect of phosgene

96 rats (weight 115–125 g) were taken and divided at random into 4 groups of 24 each. 12 rats in each group were exposed to a dosage (C_1) of 800, and the remaining 12 in each group were kept under identical conditions as controls. Five days later each group of 24 was exposed for 10 min. to concentrations of phosgene in the lethal range. The mortalities at the end of 48 hours were

Dosage (C_1) to which rats were exposed (mg.min./cu.m.)	2300	2500	3150	4400	Total
Mortality in controls	8/12	7/11	9/12	11/12	35/47
Mortality in pregassed animals	3/12	2/12	3/12	8/12	16/48

Regression lines were fitted between the mortality expressed in probits and the logarithm of the dosage (C_1) by the method described by Gaddum (1933) as elaborated by Bliss (1935 1938) and Fisher and Yates (1943).

Statistical analysis

	Degrees of freedom	χ^2	P
Differences in position of lines	1	17.6	<0.0001
Differences in slope of lines	1	0.2	0.6
Heterogeneity	4	2.2	0.7

$$\left. \begin{array}{l} \text{L(Cr)50 Pregassed 3840 mg.min./cu m} \\ \text{" Control 1880 " } \end{array} \right\} I_{\text{L(Cr)50}} = 2.0$$

Where $I_{\text{L(Cr)50}}$ is an index of effectiveness obtained by dividing the L(Cr)50 for pregassed by the L(Cr)50 for control animals

There is little doubt that when subsequently exposed to the same concentration there is a lower mortality in the pregassed than in the control animals

Phosgene and hexamine

Larger doses of phosgene for pregassing might produce greater effects, but would result in deaths in the pregassing. It was therefore decided (i) to pregas with a dosage (Cr) of 6,000, but to prevent death by administering oral hexamine* immediately beforehand, and to compare this with normal pregassing, (ii) to use intervals of both 3 and 7 days between pregassing and lethal gassing so as to compare the effect of altering the periods between gassing, (iii) to carry out the experiment with mice in order to test whether they behaved similarly to rats, and (iv) to carry out the whole experiment at two different concentrations of phosgene.

96 mice were divided at random into 8 groups of 8, and 2 of 16. The treatment applied to the groups and the resulting mortalities are shown below. The dose of hexamine used was about 2 g per kg. (0.2 c.c. of a 20 per cent (w/v) solution orally immediately prior to gassing).

2nd gassing Cr in mg.min./cu.m	Pregassed 3 days before 2nd exposure with Cr		Pregassed 7 days before 2nd exposure with Cr		No pregassing
	600	6000 and hexamine	600	6000 and hexamine	—
2,450	6/8	5/8	6/8	8/8	16/16
1,500	4/8	3/8	2/8	5/8	14/16

Statistical analysis

By a simple extension of the technique described by Bliss (1935), we can fit regression lines for the 4 treatments and control, and compare these for differ-

*Hexamine has a great chemical affinity for phosgene, and oral administration (2 g per kg) immediately prior to gassing will prevent phosgene poisoning.

ences in position and slope. The variation between slopes is not significant ($\chi^2=1.70$ for 4 degrees of freedom, $P=0.8$). Comparing the lines for position, we find

Comparison of methods of pregassing	Degrees of freedom	χ^2	P
Time (3 days v 7 days)	1	1.55	0.2
Dosage (600 v 6000 + hexamine)	1	1.55	0.2
Comparison of pregassing (in general) with controls	1	11.41	<0.001

L(Ct)50 pregassed (average)	1630 mg.min./cu.m. }	$I_{L(Ct)50} = 1.6$
L(Ct)50 control	1020 mg.min./cu.m. }	

In general pregassing of mice has a highly significant effect. There is no evidence from this experiment that the effect is different after 3 or 7 days. Increase in dosage (Ct) of the second gassing from 1,500 to 2,450 mg.min./cu.m. produces a uniform increase of probit in all the groups, and the effect is equally marked at both dosages.

Exposure in the preliminary gassing to a dosage of 6,000 after oral hexamine produces an effect no greater than that of exposure to 600 without hexamine.

Duration of the effect

In order to obtain further information on the duration of the effect, 70 mice were taken and divided at random into 7 groups of 10. One group was kept as a control and the other 6 were exposed to a phosgene dosage of 600 mg.min./cu.m. at 1, 2, 3, 5, 7, and 10 days before the second gassing. The whole 70 mice were then exposed to a dosage (Ct) of 5,850 mg.min./cu.m. (i.e., about 3 times the L(Ct)50) at the same time. The time of death of the mice was noted. We have shown elsewhere (Box and Cullumbine, 1947) that the reciprocal of survival time is closely correlated with dosage. The survival times were therefore transformed to reciprocals for analysis. The median survival times are given below together with the analysis of variance of the transformed variate. The data are graphed in Fig. 2, using the reciprocal scale.

Second gassing (Ct = 5,850, t = 10) on day Z.

Preliminary gassing on day	Z-1	Z-2	Z-3	Z-5	Z-7	Z-10	Control
Estimated median survival time (hours)	4.3	5.0	9.1	10.4	7.5	5.3	5.5

ANALYSIS OF VARIANCE OF RECIPROCAL SURVIVAL TIMES ($100 \times \text{HR}^{-1}$)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (F)
Between groups	6	1529	255	} 8.8
Within groups	63	1802	29	
Total	69	3331		

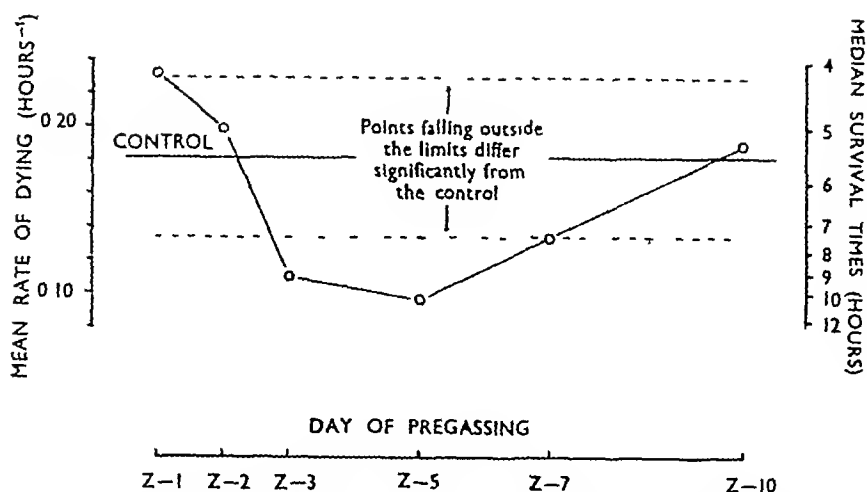


FIG 2—The median survival time (plotted as reciprocal) of mice pre-gassed at different times and exposed to phosgene (dosage $Cr=5,850$, $t=10$ min)

It is clear that the variation between groups is highly significant ($P < 0.001$) and that the animals in the group pre-gassed one day before die significantly faster, whilst those pre-gassed 3, 5, and 7 days before die significantly slower than the control animals. The index of effectiveness I_T (obtained by dividing survival time in treated group by the survival time in controls) reaches a maximum of 1.9 five days after pre-gassing.

Pregassing more than once

This experiment was designed to test whether the effect could be increased by exposure more than once to pre-gassing. The pre-gassing doses were given 12, 8, and 4 days before the dose in the lethal zone. 96 mice were used and divided at random into 8 groups, A to H, each containing 12 mice.

On day Z-12	groups C, D, G and H	were exposed to a dosage (Cr) of	600
" , Z-8	" D, B, F and H	" " "	550
" , Z-4	" E, F, G and H	" " "	600
" , Z	" A, B, C, D, E, F, G, H	" " "	final dosage of 2,850

MORTALITIES (OUT OF 12) AFTER 48 HOURS

A 11/12	B 10/12	E 7/12	F 6/12
C 11/12	D 12/12	G 5/12	H 3/12

The data may be analysed by transforming the proportions to angles using the transformation $x = \sin^{-1} \sqrt{p}$ (where p is the proportion dying). This will have the effect of stabilizing the variance. The technique was exactly as described by Fisher and Yates (1943). The expected values can in this case be obtained by inspection of the data, but in more awkward cases the graphical methods suggested by Richards (1941) have proved helpful.

Analysis of variance of the transformed variate

Effect	Degrees of freedom	Sum of squares	χ^2	P
Z-12 1st Pregassing	1	0	23.4	<0.00001
Z-8 2nd "	1	12		
Z-4 3rd "	1	1588		
Interactions				
1 x 2	1	101		
1 x 3	1	0	26.5	<0.001
2 x 3	1	101		
1 x 2 x 3	1	12		
Total	7	1814		

$$\text{Theoretical variance } \frac{820.7}{12} = 68$$

Pregassing on Z-12 and Z-8 days had no effect upon mortality, although pregassing at Z-4 produced a highly significant decrease in mortality in all groups

Lung damage and the effect

To test whether damage to the lungs was necessary to produce the effect, the following experiment was carried out. 48 rats were divided at random into 4 groups of 12, A, B, C, and D.

Group A was given a pregassing dosage on day Z-5,

B was given a pregassing dosage on day Z-5 immediately after oral hexamine

C was kept as a control,

and D was given oral hexamine *only* on day Z-5

A, B, C, and D were all exposed on day Z to a dosage of 3,150 mg.min/cu.m. After the first exposure group B showed no signs of distress. (Rats treated in this way showed no signs of pulmonary oedema at autopsy 12 and 24 hours after gassing, thus it appeared that oral hexamine was completely effective in preventing the action of this concentration of phosgene.) The 48 hour mortality after the second exposure was

	No hexamine	Hexamine
Pregassed	A 3/12	B 9/12
Not pregassed	C 9/12	D 9/12

Statistical analysis

	Degrees of freedom	χ^2	P
Pregassing with no hexamine	1	4.17	<0.05

Thus pregassing with no hexamine significantly reduces mortality, pregassing with hexamine so that no pulmonary damage is produced is ineffective. It would appear necessary therefore to cause lung damage in order to produce the effect. At first sight this experiment appears to contradict our previous experiment in which mice were gassed with a *Ct* of 6,000 after hexamine. However, in that experiment the administration of oral hexamine was not able to neutralize the whole effect of a very high dosage (6,000) of phosgene.

Duration of lung damage

In order to investigate lung damage produced after different time intervals by the pregassing dosage and to try to correlate it with the effect produced, groups of mice were exposed to the pregassing dose and autopsied after 1, 2, 3, 4, 5, 7, 10, and 14 days. The trachea was tied off before the thorax was punctured and paraffin wax sections prepared, these were examined by Professor G R Cameron, whose observations are given in Table I.

It would appear that the oedema is present for the first four days and that no damage is visible after the 10th day. When this result is compared with our experiment on the duration of the effect, the return to normality appears to correspond with a cessation of the effect, which is also absent in the first few days when oedema is most severe.

TABLE I

HISTOLOGICAL EXAMINATION OF LUNGS OF MICE AT VARIOUS TIMES AFTER PREGASSING
All mice were exposed to a dose (*Ct*) of 600 mg.min./cu m phosgene

No. of days mice killed after pregassing	No. of mice in group	Histological examination of lungs
1 day	5	All show varying degrees of oedema some with patches of leucocytic infiltration. Bronchial and bronchiolitic epithelia seem intact or in process of being lifted up by oedema. A few leucocytes.
2 days	5	Oedema seems more severe and extensive. Infiltrated with leucocytes and monocytes. Bronchial and bronchiolitic epithelia intact. Collapse.
3 days	5	Varying amounts of oedema in two cases. Severe in others. Very patchy with areas of collapse showing numerous leucocytes. Bronchial and bronchiolitic epithelia intact.
4 days	5	Two still with extensive oedema and collapsed infiltrated alveoli. Remainder with hardly any oedema. Bronchial and bronchiolitic epithelia intact, normal.
5 days	5	Two show extensive patchy oedema and collapse with emphysema. Remainder show slightly patchy oedema resolving. Bronchial and bronchiolitic epithelia intact and normal in all.
7 days	4	Three normal. One shows small patches of collapse and alveolitis. Bronchial and bronchiolitic epithelia normal.
10 days	4	All normal (one shows much recent haemorrhage. Traumatic).
14 days	4	All normal.

Differences in respiration between control and pregassed animals

When toxic substances are injected into animals the variation in response is largely due to the difference in susceptibility between them, but when animals are exposed to a gas the dose which each animal inhales is partly determined by the respiration of that animal. Experiments were made in order to find out whether the pregassing effect could be explained by differences in respiration in the pregassed and control groups during the terminal gassing. Ideally it would be useful to measure the total air breathed, the oxygen uptake, the CO_2 output, and the respiration rate, in view of the practical difficulties involved only the last two were measured.

Respiration rate

Two groups of rats were taken at random and one group exposed to the pregassing concentration. Five days later all the rats were exposed in the chamber two at a time, one rat being taken from the pregassed and one from the control group. They were placed in small wire cages close to a glass window in the gassing chamber, and their respirations counted by two observers, possible bias in the observer being eliminated by tossing a coin to decide which observer should count a particular rat.

1. In the first experiment 9 pairs of rats were exposed. Respirations were counted for 15-second periods every two minutes during the 10-min exposure. Some animals struggled and others held their breath, resulting in great variation between animals and difficulty in counting.

2. In a second experiment the rats were lightly anaesthetized with 0.5 c.c. per kg of nembutal solution. (This would itself affect the respiration rate, but it was thought that any systematic involuntary difference would still be apparent.) 13 pairs of rats were used and respirations counted for 1/2-min periods every minute during the 5-min exposure. The results for these two experiments are plotted in Fig. 3. It is clear that the animals cut

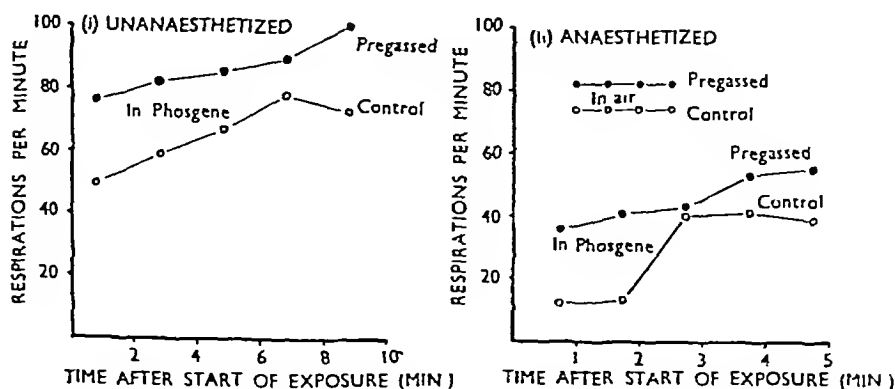


Fig. 3—The average respiration rates (respiration per min.) of control and pregassed rats during exposure to phosgene. (i) Unanaesthetized $C=500$ mg./cu.m, $t=10$ min. (ii) Anaesthetized $C=700$ mg./cu.m, $t=5$ min.

down their respiratory rate in phosgene very markedly at first (even when anaesthetized), but breath holding lessens as the exposure proceeds (Compare Boyland *et al* 1946)

TABLE II

RESPIRATION RATES PER MINUTE OF ANAESTHETIZED AND UNANAESTHETIZED RATS IN AIR AND AIR-PHOSGENE MIXTURES

	(i) Not anaesthetized $Ct = 5,000 \text{ } t = 10$		(ii) Anaesthetized $Ct = 3,500 \text{ } t = 5$		(iii) Anaesthetized $Ct = 5,900 \text{ } t = 10$	
	Control	Pregassed	Control	Pregassed	Control	Pregassed
Average respiration rate in air	160*	—	74	82	—	—
Average respiration rate in COCl_2	66	86	32	46	41	49
Mortality	9/9	9/9	†4/13	2/13	10/10	4/8
Percentage increase in respiratory rate in pregassed group	30		44		19	
t_f = 'Students' ratio for f degrees of freedom	$t_{14} = 2.10$ $P = 0.052$		$t_{14} = 3.31$ $P = 0.003$		$t_{18} = 1.33$ $P = 0.200$	
	Combined $P < 0.005$					

*Rate obtained from Fig. 54 given by Gaddum (1940)

†In the anaesthetized groups the mortalities were lower than would be expected normally, presumably because of the reduction in breathing rate caused by anaesthesia

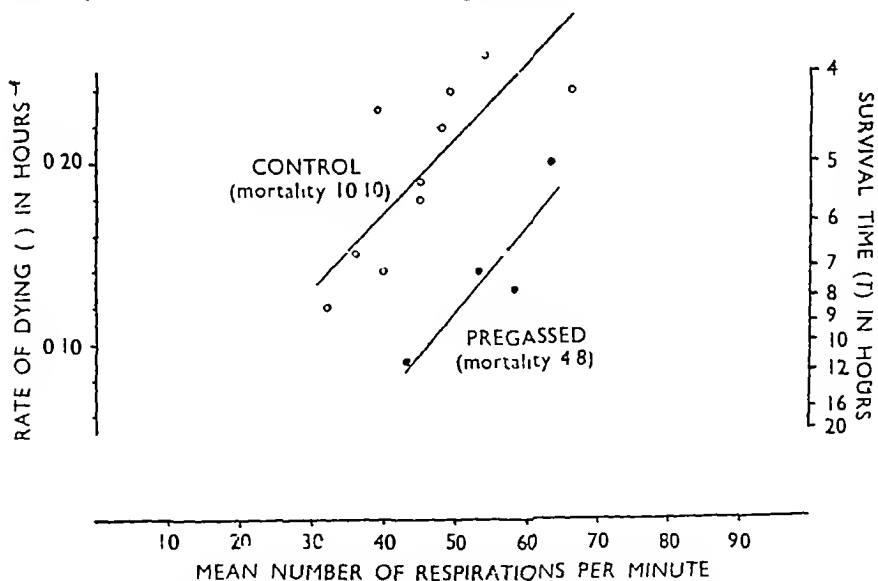


Fig 4—A comparison of the respiration rates of anaesthetized rats during exposure to phosgene and their subsequent survival times, in control and pregassed groups $C = 590 \text{ mg/cu.m}$, $t = 10 \text{ min}$

3 In a third experiment an exposure time of 10 min was used, otherwise it was similar in detail to the second experiment. There were originally 10 rats in each group but 2 died in the pregassing group under anaesthesia.

A summary of the results from these three experiments is given in Table II. The analysis shows that there is little reason for doubt that when exposed to phosgene the pre-gassed rats breathe faster than the controls.

In the third experiment each rat was marked and its survival time recorded, the results are given in Table III and are plotted together with the lines of best fit in Fig. 4.

TABLE III

AVERAGE NUMBER OF RESPIRATIONS PER MINUTE IN PHOSGENE AND SUBSEQUENT SURVIVAL TIMES OF CONTROL AND PREGASSED RATS

Control group		Pregassed group	
Average No of respirations per min in COCl_2 *	Survival time (hours)	Average No of respirations per min. in COCl_2 *	Survival time (hours)
32	8.4	18	Survived
36	6.8	40	Survived
39	4.4	43	11.7
40	7.2	53	7.0
45	5.3	54	Survived
45	5.4	58	7.8
48	4.6	63	5.0
49	4.2	64	Survived
54	3.8		
66	3.9		
Mortality 10/10		Mortality 4/8	

*In order of magnitude to facilitate reference to Fig. 4

Clearly there is a very strong correlation in the groups between survival time and respiration rate ($r=0.83$, $P<0.001$). The regression equation for the control group suggests a relationship of the type $\frac{1}{T}=KX$ where T is the survival time and X the respiration rate. Analysis of the transformed variate ($100 \times \text{hours}^{-1}$) gives

Analysis of transformed variate ($100 \times \text{hours}^{-1}$)

Source	Degrees of freedom	Sum of squares	Mean square	F
About lines	10	88.4	8.8	} 25
Between lines { slope position	1	0.4	225.0	
	1	225.0		

It is clear that there is a highly significant difference in position between the lines ($P<0.001$).

CO₂ output during gassing

The method used was similar to that described by Gaddum and Hetherington (1931) The apparatus is sketched in Fig 5

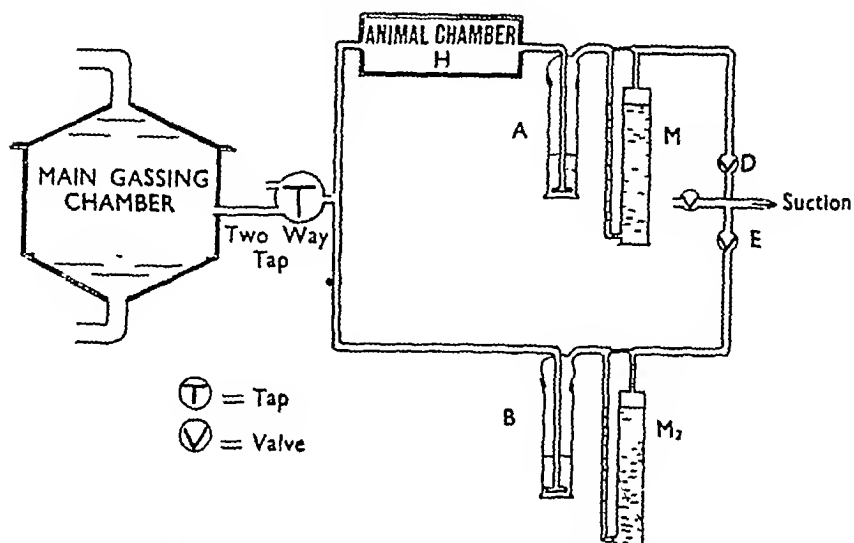


FIG 5—Apparatus for measuring CO₂ output of small animals during gassing. The flow of air along tubes E and D was adjusted to 3 litres per min. by means of the valves and the flowmeters M₁ and M₂. H was a chamber sufficiently large to accommodate the animals or animal, without too much dead space. A two-way tap allowed circuit to be opened to outside air or to a main gassing chamber. Bubbler A sampled the atmosphere together with expired air from animals, bubbler B sampled atmosphere only

The amount of CO₂ produced by the rat was calculated by deducting the CO₂ contained in the air—measured by bubbler B—from that produced by the rat plus the amount contained in the air—measured by bubbler A

In order to estimate the actual concentrations of phosgene to which the animals were exposed (and not that in the large chamber), a method was devised whereby CO₂ and phosgene could be absorbed and estimated in the same bubbler. Our methods of overcoming certain problems arising in these measurements are discussed in the Appendix

It was first of all necessary to know whether the CO₂ output during exposure to COCl₂ and the dose of phosgene breathed were related. An experiment was therefore carried out in which normal rats were exposed to lethal concentrations of phosgene one at a time. The CO₂ output and phosgene concentrations were estimated and the survival time of each animal recorded. The results are given in Table IV

It might be expected that most simply the dose breathed would be proportional to the CO₂ output (*R*). The dose breathed cannot be measured, but we have found (Box and Cullumbine, 1947) that the median mortality time and *Ct* are related by the type of expression $\frac{1}{T} = K (Ct)$ where *T* is the survival time,

TABLE IV

CO₂ OUTPUT OF RATS IN PHOSGENE AND THEIR SUBSEQUENT SURVIVAL TIMES

(1)	(2)	(3)	(4)	(5)	(6)
Mean concentration <i>C</i> in mg./cu.m. phosgene	C.c. of CO ₂ produced per kg. body wt. in phosgene in 10 min.* (<i>R</i>)	Fractional CO ₂ output of average <i>R</i> - 199	Survival time (hours)	Reciprocal survival time (hours ⁻¹)	(1) × (3) "C" when differences in <i>R</i> are allowed for
458	87	0.44	about 24	0.042	202
436	108	0.54	survived	—	235
427	127	0.64	14.5	0.069	273
496	138	0.69	19.0	0.053	342
499	143	0.72	about 24	0.042	359
442	154	0.77	11.0	0.091	340
475	155	0.78	about 24	0.042	370
442	182	0.92	9.3	0.107	407
541	189	0.95	6.6	0.152	514
386	193	0.97	survived	—	374
449	198	0.99	19.5	0.051	445
894	212	1.07	12.2	0.082	957
446	217	1.09	9.7	0.103	486
449	228	1.15	5.3	0.189	516
479	284	1.43	5.2	0.192	685
464	360	1.81	7.0	0.143	840
522	411	2.06	4.4	0.227	1075
	Mean of <i>R</i> - 199				

*Rats in order of CO₂ output.

C is the concentration to which the animal is exposed for time *t*, and *K* is constant. Hence the type of expression which might be expected would be

$$\frac{1}{T} = KCR \quad (i)$$

where *R* = c.c. of CO₂ produced per kg body weight during the period of gassing

If this were true, converting to logs we should have

$$\log \frac{1}{T} = K + b_1 \log C + b_2 \log R \quad (ii)$$

and *b*₁ and *b*₂ would both be equal to unity

To test this, the values of $\frac{1}{T}$, *C*, and *R* were converted to logarithms. The best fitting equation obtained by the method of least squares was

$$\log \frac{1}{T} = -5.13 + 0.56 \log C + 1.13 \log R$$

The values for *b*₁ and *b*₂ and their standard errors are

$$0.56 \pm 0.58 \text{ and } 1.13 \pm 0.26 \text{ respectively}$$

The range of values of *C* here is very small and consequently the coefficient of log *C* (0.56) is very unreliable and not significantly different from zero or from unity. But our previous work leads us to expect that this part of equation (ii) is well founded and the true value of the coefficient *b*₁ will not be far from the expected value, i.e., unity. The coefficient *b*₂=1.13 is highly significantly

different from zero and not significantly different from one. Hence there are grounds for believing that the relationship is of type (i)

The experiment leaves no doubt as to the correlation between CO_2 output and reciprocal survival time ($r=0.79$). As the dosage (Ct) was not always constant, the partial correlation coefficient between reciprocal survival time and CO_2 output with the effect of C eliminated was worked out, this is still 0.79 correct to two places of decimals, a value which is highly significant.

It is clear, therefore, that the CO_2 output during exposure to phosgene gives a good indication of the amount of gas breathed. The experiment shows that there is an enormous difference in the amount of gas breathed by different animals, presumably owing to the variations in breath-holding and in the degree of activity of the animals in the concentration. In fact, over half the variation ($R^2=0.65$) is attributable to factors involved in differences of respiration and concentration of the gas. The remaining variation ($1-R^2=0.35$), or less than half of it, is attributable to other factors such as differences of susceptibility. In order to test the hypothesis that CR represented the dose breathed, columns (1) and (3) of Table IV were multiplied to give a product which might be considered as

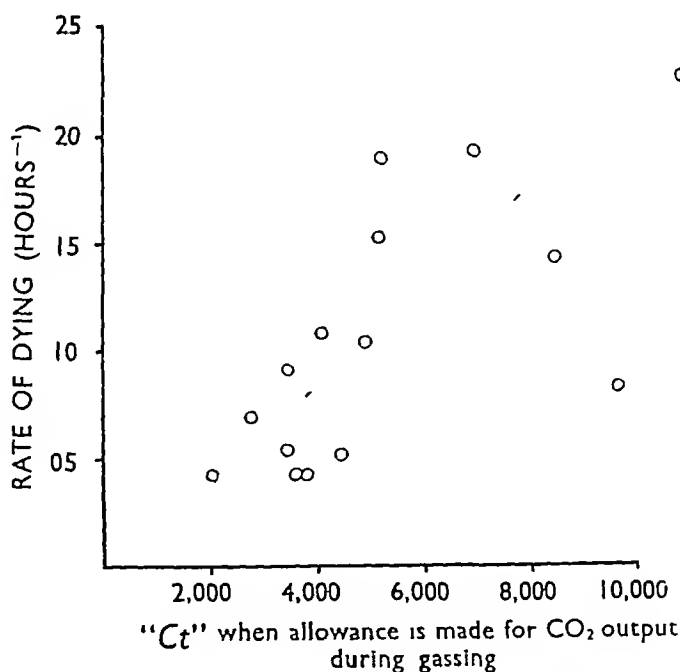


FIG 6—The points represent the survival times (plotted as reciprocals) for corresponding values of Ct for individual animals when allowance is made for differences in CO_2 output during the gassing. The line is that obtained in a previous investigation for median survival time at various dosages.

representing better the "dosage" to which the lungs of animals were exposed In Fig 6 reciprocal survival time is plotted against this product multiplied by t (10 min) and the points are compared with the relationship between Cr and survival time found before (Box and Cullumbine, 1947) It can be seen that the hypothesis is in reasonable agreement with the facts

CO₂ output of control and pregressed rats during gassing

Twenty rats, which had been submitted to the pregressing concentration 5 days previously, and 20 controls were exposed to phosgene in the apparatus in groups of 4 at a time The CO₂ production was first determined during a 10-min period while the animals were breathing air and later during a 10-min period while they were breathing an air-phosgene mixture of mean $Cr=1450$ ($t=10$ min) The results were as follows

Control				Pregassed			
No of rats	Average weight (g.)	Average rate of CO ₂ production (c.c./min./kg)		No of rats	Average weight (g.)	Average rate of CO ₂ production (c.c./min./kg.)	
		In air	In COCl ₂			In air	In COCl ₂
4	197	32.4	16.0	4	207	22.6	16.0
4	195	24.3	15.6	4	202	29.5	17.8
4	197	26.8	16.2	4	210	31.0	20.3
4	200	25.4	14.9	4	190	18.3	16.7
4	197	32.4	18.9	4	190	30.9	24.1
Means		28.2	16.3	Means		26.4	19.0
Mortality		1/20		Mortality		0/20	

The apparent increase in CO output in phosgene (16.3 to 19.0 c.c. CO₂/min./kg) is not statistically significant, $t=1.64$ for 8 degrees of freedom and P is between 0.2 and 0.1

The dosage used in the previous experiment was rather low, so the experiment was repeated using higher concentrations Eight pregressed and 8 control rats were used in the experiment and they were exposed in pairs

Control				Pregassed			
No of rats	Average wt (g.)	Cr(mg./min./cu.m.)	c.c. CO ₂ /min./kg	No of rats	Average wt (g.)	Cr(mg./min./cu.m.)	c.c. CO ₂ /min./kg
2	185	1,940	23.4	2	182	2,270	32.4
2	185	3,360	20.4	2	195	3,360	23.1
2	210	2,610	23.4	2	190	2,390	20.5
2	190	3,700	23.3	2	185	3,690	20.3
Means	192	2,900	22.6	Means	188	2,780	24.1
Mortality		6/8		Mortality		3/8	

As before the apparent increase in CO production (22.6 to 24.1 c.c. CO₂/min./kg) is not statistically significant $t=0.5$ for 6 degrees of freedom and $P=0.6$

It must be concluded that there is no significant difference in CO₂ production of pregressed and control rats during subsequent exposure to phosgene

The respiration experiments suggest that after pregassing and during the period when oedema has subsided but visible lung damage persists (i.e., from about the 4th–7th day), the animals breathe more rapidly but less deeply. It seems likely that this would result in the effect demonstrated, since damage in the second gassing would probably be more superficial with this type of breathing.

SUMMARY AND CONCLUSIONS

- 1 When rats and mice are exposed to preliminary non-lethal doses of phosgene a transitory effect (lasting from about the 3rd to the 7th day in mice) is produced, resulting in an apparent increase of resistance to phosgene
- 2 In order to produce this effect it is necessary to produce lung damage
- 3 Repeated exposures do not produce a cumulative effect
- 4 The respiration rate is related to the dose breathed
- 5 Rats which have been exposed to a pregassing dose breathe more rapidly in phosgene, but take longer to die
- 6 The CO_2 output is related to the dose breathed
- 7 There is no significant difference between the CO_2 outputs of pregassed and control rats during exposure to phosgene 5 days later

It seems likely that the effect can be explained by the more rapid and shallower type of breathing of the pregassed rats in phosgene caused by lung damage in the first exposure.

APPENDIX

Problems arising in the measurement of CO_2 production of rats and mice during exposure to phosgene (or other toxic agents)

General

The normal amount of CO_2 present in laboratory air is about 0.05 per cent or 0.5 c.c. per litre. The CO_2 output of an average size rat is about 4 c.c. per min. Hence in the type of apparatus described the greater the air flow used the greater will be the proportion of CO_2 absorbed from the air and the less the proportion due to the rat. Hence the air flow must not be too high, otherwise atmospheric CO_2 will tend to swamp the CO_2 expired by the rat and reduce the accuracy of the method. On the other hand, in order that the air already in the rat chamber may be washed out rapidly, the dead space must be cut to a minimum and flow kept as high as possible. Further, the type of bubbler with sintered glass diffuser plates cannot be used at flows much above 3 litres/min without frothing.

Bearing these considerations in mind, the following design was adopted

- 1 Not more than 1 litre of chamber space per rat (dead space about 750 c.c.)
2. Flow of 3 litres/min
- 3 Phosgene and CO_2 were sampled in the same bubbler

With this arrangement the chamber would be completely washed out in a quarter of a minute and the error would be negligible in 10-min exposures. Further, for every 4 c.c.

of CO_2 produced by the rat only about 1.5 c.c. would pass through the bubbler from the air. The estimation of CO_2 and phosgene in the same bubbler (1) ensures that the actual atmosphere breathed by the rat is sampled, (2) gives double the rate of flow possible with two bubblers using the same rate through the chamber, and (3) avoids duplication of solutions, bubblers, flowmeters, and air lines.

The absorbent was a mixture of 25 c.c. of $N/1$ NaOH and 50 c.c. of 6 per cent (w/v) hexamine. The bubblers were Dreschel bottles with sintered glass diffuser plates.

Method

The contents of the bubbler were washed out into a beaker and 50 c.c. of 66 per cent (w/v) solution of barium nitrate added to precipitate the carbonate. The volume was made up to 170 c.c. and a few drops of phenolphthalein added. The residual alkali was titrated with $N/1$ nitric acid. The endpoint (red-colourless) is extremely sharp, as the precipitated barium carbonate acts as a white background for the colour change. The hydrogen ion concentration was then adjusted to $\text{pH } 2$ by adding sufficient $N/1$ HNO_3 to make the total volume added 48.5 c.c.

The solution remaining was colourless and the chloride was estimated by titrating with $M/50$ mercuric nitrate, using 1.5 c.c. of 1 per cent diphenylcarbazone in alcohol as indicator (Roberts, 1936). The large amount of excess acid necessary was due to the buffering action of the hexamine.

It was found by using a pH meter that the addition of the amounts of reagents described always resulted in the correct pH . Blanks were carried out for both titrations using all the reagents. It was necessary to make a correction for the CO_2 produced by the breakdown of phosgene, this correction was in practice very small, and is described below.

The efficiency of absorption of phosgene

As the procedure described above and the concentration of the reactants were not quite the same as we normally used for sampling and analysing phosgene concentrations, tests were carried out as follows: a phosgene-air mixture in which the COCl_2 was about 1,000 mg/cu m (which is higher than any concentration used in the experiment) was sucked through two bubblers in series, using the absorbent mixture described, in a set of three experiments no chloride was detected in the second bubbler.

The efficiency of absorption of CO_2

The first experiments were carried out with ordinary bead bubblers but the efficiency of absorption of CO_2 with 15 c.c. in each bubbler and rates of flow of 3–5 litres/min was in no case greater than 50 per cent. In order to increase the efficiency larger amounts of the reagents and Dreschel bottles with sintered glass diffuser plates were used. With a total of 75 c.c. of reagents, efficiencies of over 80 per cent with a rate of flow of 3 litres/min were obtained.

It was found that owing to small physical differences the efficiencies of the bubblers varied. Each bubbler had therefore to be standardized and its efficiency measured.

This can be done (1) if the exact concentration of CO_2 bubbled through is known or (2) by using a long train of bubblers so that the leak at the end is negligible. However, the exact determination of the CO_2 without the use of bubblers is not an easy task and the use of a long train of bubblers is clumsy and involves a large number of titrations also the back pressure set up by these bubblers is high, and this causes added complications. In view of the above the following device was used.

If the efficiency of absorption is independent of the CO_2 concentration imagine two bubblers of unequal efficiency, arranged in series.

rats, bubbler (2) in position B absorbed CO_2 from the air only Expected phosgene concentration 500 mg/cu.m Duration of run 10 min

Bubbler A (rats and air)		Bubbler B (air only)	
COCl_2 titration	CO_2 titration	COCl_2 titration	CO_2 titration
c.c. of $M/50 \text{ Hg}(\text{NO}_3)_2$	c.c. of $N \text{ HNO}_3$	c.c. of $M/50 \text{ Hg}(\text{NO}_3)_2$	c.c. of $N \text{ HNO}_3$
Blank 0.10	24.80	0.10	24.80
Test 14.50	12.65	15.80	23.50
Difference 14.40	12.15	15.70	1.30

COCl_2 1 ml. of $M/50 \text{ Hg}(\text{NO}_3)_2 \equiv 0.99 \text{ mg. COCl}_2$
 average titration $\frac{A+B}{2} = 15.05$ Concentration = $\frac{15.05 \times 0.99 \times 1000}{30} = 497 \text{ mg./cu m}$
 Hence $Ct = 4,970 \text{ mg.min./cu.m}$

CO_2 correction

In the hydrolysis of COCl_2 , CO_2 will be formed Correction is made for this here but as may be seen the effect of this correction is normally so small that it may be ignored

If x is the COCl_2 titration (c.c. of $M/50 \text{ Hg}(\text{NO}_3)_2$) then

correction of CO_2 will be simply $\frac{x}{50}$ c.c. N acid.

CO_2 1 c.c. N acid $\equiv 11.2 \text{ c.c. CO}_2$

Total CO_2 through bubbler A $\equiv \frac{12.15}{0.85} = 14.30 \text{ c.c. } N \text{ acid}$

Correction for COCl_2 $\equiv \frac{14.4}{50} = 0.28 \text{ c.c. } N \text{ acid}$

Difference (CO_2 from rat and air) $\equiv 14.02 \text{ c.c. } N \text{ acid}$

Total CO_2 through bubbler B $\equiv \frac{1.30}{0.82} = 1.58 \text{ c.c. } N \text{ acid}$

Correction for COCl_2 $\equiv \frac{15.7}{50} = 0.31 \text{ c.c. } N \text{ acid}$

Difference (CO_2 from air only) $\equiv 1.27 \text{ c.c. } N \text{ acid}$

Hence CO_2 from 4 rats $\equiv 14.02 - 1.27 = 12.75 \text{ c.c. } N \text{ acid}$

and this is equivalent to 143 c.c. CO_2

Hence CO_2 output per rat was 3.57 c.c./min.

Our thanks are due to the Director-General of Scientific Research (Defence) for permission to publish these results, and to our colleagues at Porton for their advice and criticism Our special thanks are due to Prof C R Cameron for his help and encouragement.

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THE CHOLINESTERASE INHIBITING ACTION OF FLUOROPHOSPHONATES

BY

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From the Physiological Laboratory Cambridge

(Received November 18 1946)

Recently the cholinesterase inhibiting action of fluorophosphonates has been used for physiological experiments and in clinical trials. Our knowledge that these compounds had such an action dates back to experiments carried out in this laboratory during the early part of the war, but the results could not be published at that time. The first compound tested was the dimethyl fluoro phosphonate, and its cholinesterase inhibiting action was found in 1941 (Adrian, Feldberg, Kilby, and Kilby). In 1942 the more toxic diisopropyl fluorophosphonate was prepared by McCombie and Saunders and found to have the same action (Barrett, Feldberg, Kilby, and Kilby). Since then various workers have examined in detail the cholinesterase inhibiting activity of this and other related fluorophosphonic esters.

The pharmacological action of the fluorophosphonates when injected into animals or inhaled by men or animals suggested the possibility of such mode of action. The pharmacological effects of these compounds will be described shortly (Kilby and Kilby). The most conspicuous effect in man on exposure to the vapours of these substances was a long-lasting miosis associated with a spasm of accommodation. The effect started a few minutes after the exposure and lasted for a few days, or even up to a week in the case of the diisopropyl ester. In the absence of other central effects in man on exposure to these compounds in low concentrations, it seemed unlikely that the effects were due to a central action. They suggested rather a local effect on the eye by absorption through the mucous membrane, and this was shown by the fact that if one eye was protected from the vapour, the pupil of that eye did not constrict. A peripherally produced miosis of such long duration at once suggested the possibility that these compounds might act not directly on the smooth muscles in the eye, but indirectly, like eserine, by inhibiting the action of cholinesterase.

To test this possibility we first examined the effect of fluorophosphonates on the isolated rabbit's intestine. On such a preparation the action of drugs which, like acetylcholine, act directly on the muscle differs characteristically from that of those which, like eserine, act by inhibition of cholinesterase activity. The

directly acting drugs produce an immediate contraction which proceeds rapidly to a maximum, and after the drug has been washed out the muscle again quickly relaxes. The contraction produced by cholinesterase inhibiting drugs, such as eserine, is characterized by its long latency, its slow development, and its very gradual disappearance when the drug is washed out. The fluorophosphonates when tested on the isolated rabbit's intestine produced a contraction resembling in its details that produced by eserine and not that produced by acetylcholine. The contraction produced by the diisopropyl ester, for instance, persisted for hours after washing out the drug. We therefore proceeded to test the effects of these compounds on the activity of plasma cholinesterase.

METHODS

The cholinesterase inhibiting activity of the fluorophosphonates was compared quantitatively with that of eserine sulphate, the procedure being as follows. To 0.2 c.c. of heparinized human plasma was added 0.5 c.c. of a solution containing either eserine or the fluorophosphonate in varying concentrations, then the mixture was kept at room temperature for 10 minutes before 1 μ g. acetylcholine in 1 c.c. saline solution was added. After 5 minutes standing at room temperature, the mixture was made up to 10 c.c. with frog saline containing eserine 1 in 100,000, which at once stops the action of any cholinesterase not yet inactivated. The solution was then assayed for acetylcholine on the frog rectus muscle preparation.

RESULTS

Both the dimethyl and diisopropyl ester were found to inhibit the cholinesterase activity of human plasma, and their action was stronger than that of eserine tested in a similar way. Of the two esters, the diisopropyl had a more powerful cholinesterase inhibiting action than the dimethyl ester. An accurate quantitative comparison was made of the action of the diisopropyl ester with that of eserine sulphate. It was found that this ester in a concentration of 1 in 80 millions had a more powerful action than eserine 1 in 15 millions, but a weaker action than eserine 1 in 14 millions. Therefore, under the conditions of our experiment, the ester at 1 in 80 millions had about the same cholinesterase inhibiting action as eserine sulphate at 1 in $14\frac{1}{2}$ millions, i.e., the diisopropyl ester was about $5\frac{1}{2}$ times as active as eserine sulphate when compared weight for weight, which is about 3 times as active when compared in molar solutions.

DISCUSSION

Our results show that the fluorophosphonates have a cholinesterase inhibiting action which is stronger than that of eserine. The pharmacological actions of these compounds as far as they resemble those of acetylcholine, therefore, can be explained by the inhibition of cholinesterase and an accumulation of acetylcholine.

Dixon *et al* (1942, 1944), when later on comparing the cholinesterase inhibiting action of the diisopropyl ester with that of eserine, found that the difference

between the activity of the two was much greater than in our experiments. This discrepancy may well be due to the difference in the procedure adopted by Dixon *et al*, because the cholinesterase preparations were exposed to the action of the drugs for longer periods than in our experiments. We know, however, that eserine is slowly destroyed by cholinesterase, so that its action would diminish over longer periods. Fluorophosphonates apparently cause an irreversible inhibition, as shown by Dixon *et al*.

SUMMARY

The dimethyl and diisopropyl fluorophosphonates have a strong cholinesterase inhibiting action. This action explains the acetylcholine-like effects of these substances. The dimethyl ester was the first to be investigated (1941). Subsequent work on other compounds of the series has confirmed their action.

We are indebted to the Director-General of Scientific Research (Defence) for permission to publish the above, and to Dr H. McCombie, Dr B C Saunders, and their research team for supplying the diisopropyl fluorophosphonate used.

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THE POSSIBILITY OF TOXIC EFFECTS FROM 2,3-DIMERCAPTOPROPANOL IN CONDITIONS OF IMPAIRED RENAL OR HEPATIC FUNCTION

BY

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If 2,3-dimercaptopropanol (BAL) is to be adopted as a remedy for arsenical and other forms of poisoning, it is necessary that hazards associated with its use be understood. Toxic effects from overdosage with BAL have already been defined (Peters, Stocken, and Thompson, 1945; Waters and Stock, 1945), and the minimal lethal dose of numerous samples determined. Appreciation of such risks has led to the dose for human use being placed well below the fatal level. But there remains a problem which, so far as we know, has not been considered: the possibility of harmful effects in individuals who are suffering from impaired function of vital organs. Two such instances at once come to mind: damage to the kidneys and the liver. Both organs are concerned in the elimination and detoxication of drugs and poisons, and though their action on BAL is not clear it is likely they are important therapeutic safeguards. We have therefore carried out experiments in which the toxicity of BAL for normal animals has been compared with that for animals suffering from severe renal and hepatic damage. Such complications are frequent with arsenical poisoning and the conditions against which arsenic is employed therapeutically, in these disturbances BAL should be of value.

METHODS

Fully grown male and female rabbits and rats were used. The former averaged 2 kg body weight, the latter 200 g. Pregnant females were avoided. All were kept on the standard laboratory diet throughout the experimental period and were given water. Severe renal disease in both rabbits and rats was produced by subcutaneous injection of 5 mg./kg. uranium acetate in aqueous solution, hepatic damage in rabbits by subcutaneous injection of 1 c.c./kg. redistilled carbon tetrachloride and in rats by injection of 6 c.c./kg. of this compound. Preliminary studies showed that the uranium acetate induced massive destruction of renal tubules and evidence of serious renal functional impairment in 48 hours, which proved fatal in 5-9 days (one example is given in Table I). Carbon tetrachloride led to severe liver destruction within 24 hours, which resulted in the death of about 20 per cent

of the animals in a week after injection Uranium acetate thus produces serious renal disease and hardly any liver damage, carbon tetrachloride gives extensive liver destruction but scarcely any renal change

TABLE I
BODY WEIGHT, BLOOD UREA, AND URINARY FINDINGS IN A RABBIT WITH
URANIUM ACETATE INTOXICATION NO TREATMENT

Time interval	Body weight (kg.)	Blood urea (mg. per cent)	Urine				
			Amount excreted in 24 hours (c c)	Total urea in 24 hours (g.)	Specific gravity	Albumin	Microscopic findings
<i>Normal period</i>							
1st day	2.75	28.5	125	2.3	1022	Nil	Nil
2nd day	3.10	27.0	143	2.02	1020	Nil	Nil
3rd day	2.90	23.5	246	3.1	1018	Nil	Nil
4th day	2.82	34.0	370	3.5	1016	Nil	Nil
5th day	2.82	34.0	175	1.74	1020	Nil	Nil
6th day	2.80	34.5	160	2.17	1022	Nil	Nil
<i>Uranium acetate (5 mg/kg) given on 6th day</i>							
7th day	2.90	37.0	185	2.87	1022	Present	Nil
8th day	2.80	73.0	215	2.34	1024	Present	Red blood cells
9th day	2.70	159.0	38	0.37	1030	Present ++	Red blood cells casts
10th day	2.70	300.0	Nil	0.37	1030	Present ++	Red blood cells casts
11th day	2.60	348.0	Nil	0.37	1030	Present ++	Red blood cells casts
13th day	2.20	288.0	5	0.005	1030	Present ++	Granular casts
14th day	2.30	376.0	4	0.14	1030	Present	Granular casts
15th day	Dead						

Two, three, and four days after the injection of uranium acetate and one day after carbon tetrachloride, BAL (in the form of BAL 4 g benzylbenzoate 8 c.c., peanut oil to 50 c.c.) was injected intramuscularly into groups of 10 or 15 such animals, in doses varying from 60 to 120 mg/kg. Equal groups of normal animals were injected in parallel with similar doses of BAL. Since it is a matter of experience that deaths from BAL poisoning occur within a few hours of administration we have accepted a time limit of 24 hours and attributed all deaths taking place during that period to BAL. In most animals death resulted within 4 hours, which is the experience of other workers. Toxic symptoms were carefully noted. Post-mortem examination of fatal cases and microscopic examination of the chief organs, especially of liver and kidneys, were carried out on most of the animals. Material was fixed in 10 per cent formal alcohol, embedded in paraffin and sections stained with Ehrlich's acid haematoxylin and eosin, Weigert's iron haematoxylin and Van Gieson, Heidenhain's azan method for kidneys. Frozen sections were also prepared in some cases and stained for fats.

RESULTS

Tables II and III summarize the experiments

1 Toxicity of BAL in animals with severe renal damage

Table II shows that there is no difference in reaction to BAL between rabbits and rats with severe renal damage developing two days after injection of uranium acetate and normal animals. Mortality rates are closely in agreement in both series. Toxic symptoms are similar and seldom occur in animals other than those dying from BAL intoxication. In other words, the presence of serious renal disturbance

TABLE II

TOXICITY OF BAL IN ANIMALS WITH RENAL IMPAIRMENT 2, 3, AND 4 DAYS AFTER ADMINISTRATION OF URANIUM ACETATE. DEATHS DURING THE 24-HOUR PERIOD FOLLOWING INJECTION OF BAL

Dose of BAL (mg./kg.)	No of animals	No dying	Per cent mortality	No showing toxic symptoms (percentage in brackets)	Dose of BAL (mg./kg.)	No of animals	No dying	Per cent mortality	No showing toxic symptoms (percentage in brackets)
<i>'Renal damage' group</i>					<i>Normal group</i>				
RABBITS									
<i>(i) Two days after subcutaneous injection of uranium acetate (5 mg/kg)</i>									
100	15	14	93	15 (100)	100	15	13	87	15 (100)
80	15	8	53	9 (60)	80	15	8	53	9 (60)
60	15	3	20	3 (20)	60	15	2	13	3 (20)
40	15	0	0	0 (0)	40	15	0	0	0 (0)
<i>(ii) Three days after subcutaneous injection of uranium acetate (5 mg/kg)</i>									
100	10	10	100	10 (100)					
80	10	10	100	10 (100)					
60	10	5	50	5 (50)					
40	10	1	10	1 (10)					
<i>(iii) Four days after subcutaneous injection of uranium acetate (5 mg/kg)</i>									
100	10	10	100	10 (100)					
80	10	9	90	10 (100)					
60	10	5	50	5 (50)					
40	10	1	10	1 (10)					
RATS									
<i>Two days after subcutaneous injection of uranium acetate (5 mg/kg)</i>									
140	10	10	100	10 (100)	140	10	8	80	10 (100)
120	10	8	80	10 (100)	120	10	5	50	5 (50)
100	10	4	40	4 (40)	100	10	2	20	2 (20)
80	10	0	0	0 (0)	80	10	0	0	0 (0)

does not alter the LD50 or lead to unexpected signs of BAL intoxication. So far as we can judge, renal upset is not increased by BAL, nor can we find any microscopical evidence of more severe renal structural changes in that group. Since few animals survived the experiment, we have not attempted an analysis of the influence of BAL on the survival time of nephrotic subjects.

Rabbits given BAL three and four days after injection of uranium acetate show somewhat decreased tolerance for BAL (Table II) Thus 50 per cent of animals in both groups died after 60 mg/kg BAL as against a fatal level of 80 mg/kg with normal animals. The LD₅₀ has thus been decreased a little by the damage to the kidneys. At this stage the animals were very ill, their renal function being grossly impaired as indicated by the very small amount of urine excreted, the high blood urea, and the severe destruction of renal structure shown microscopically. Reference to Table I brings out these features. On the fourth day of uranium poisoning there is often complete suppression of renal function and the animals may be moribund. It is therefore reassuring that, despite such severe renal disturbance, the toxicity of BAL is not greatly increased.

TABLE III

TOXICITY OF BAL IN ANIMALS WITH HEPATIC IMPAIRMENT PRODUCED BY INJECTION OF CARBON TETRACHLORIDE DEATHS DURING THE 24-HOUR PERIOD FOLLOWING INJECTION OF BAL

Dose of BAL (mg/kg.)	No of animals	No dying	Per cent mortality	No showing toxic symptoms	Per cent showing toxic symptoms	Dose of BAL (mg/kg.)	No of animals	No dying	Per cent mortality	No showing toxic symptoms	Per cent showing toxic symptoms
<i>"Liver damage" group</i>						<i>Normal group</i>					
<i>24 hours after carbon tetrachloride (1 c.c./kg.)</i>											
80	10	9	90	10	100	80	15	8	53	9	60
60	10	2	20	9	90	60	15	2	13	3	20
40	10	1	10	8	80	40	15	0	0	0	0
<i>24 hours after carbon tetrachloride (6 c.c./kg.)</i>											
120	10	6	60	10	100	140	5	4	80	5	100
100	10	6	60	10	100	120	15	4	27	4	27
80	10	1	10	8	80	100	15	0	0	3	20
60	10	0	0	6	60	80	15	0	0	0	0
						60	15	0	0	0	0

2 Toxicity of BAL in animals with severe liver damage

Table III shows an increased mortality (90 per cent) in the rabbits with liver damage during the 24 hours after administration of 80 mg/kg BAL as compared with the normal group (53 per cent). This difference is statistically significant. A difference is present too in the experiment with rats. In the "hepatic damage" group, 6 of 10 rats (60 per cent) receiving 120 mg/kg BAL died during the 24-hour period as against 4 of 15 rats (27 per cent) in the normal group. With 100 mg/kg BAL 6 of 10 of the hepatic damage group died as against none of 15 normal animals. An even more striking feature of the hepatic damage group

is the development of toxic symptoms in the animals receiving low doses of BAL, the normal controls with the same dose of BAL showing no such symptoms. No microscopic evidence of an effect on the structural damage in the liver, attributable to BAL, was obtained, but the deaths occurred in so short a time after giving BAL that this is not surprising. It seems from these experiments that a somewhat increased toxic activity may be expected when BAL is administered to animals suffering from serious liver damage. It may be conjectured that the normal liver plays a part in detoxication of BAL.

CONCLUSIONS

By means of experiments on rabbits and rats we have tried to find out whether untoward effects may arise from the use of BAL in conditions of impaired function of the kidneys and liver. Renal destruction was produced with uranium acetate (Boycott and Douglas, 1914-15) in doses sufficient to damage severely or destroy the greater part of the tubular system, the glomeruli apparently escaping. Carbon tetrachloride was used to damage the liver in quantities which destroyed at least one half, and more often two thirds, of the liver cells (Cameron and Karunaratne, 1936). In both instances the injurious actions were confined to the organ specified, though there is some evidence that other tissues are also damaged in these intoxications, our dosages apparently succeeded in checking such additional effects. When BAL was injected into animals with severe renal disease no evidence of more severe toxic effects than in normal animals was obtained until the animals were suffering from complete suppression of renal function. Even then the effect was not striking. With hepatic disturbance, however, there was enhanced toxicity of BAL, though here again lowered resistance was not pronounced. It was shown by the occurrence of toxic symptoms with low doses of BAL (40 mg/kg in rabbits and 60 mg/kg in rats), though seldom of death. Clearly, our experiments suggest that BAL should not give rise to anxiety when used in conditions of renal impairment unless this is very severe, but that caution should be exercised if there is reason to suspect hepatic insufficiency. Nevertheless, the latter hazard need not contraindicate the careful use of BAL, since tolerance is not unduly diminished in conditions of severe liver damage. We would venture the opinion that BAL might have an unfavourable action on the course of hepatic diseases if used light-heartedly without careful attention to the patient's condition. We gather from Professor R. A. Peters that he has formed a similar impression from his study of the use of BAL in the treatment of infectious hepatitis.

SUMMARY

- 1 The toxicity of BAL for animals with severe renal or hepatic damage has been compared with that in normal animals.
- 2 Animals with renal damage differed in no way from normal controls in their response to large doses of BAL. With complete or almost complete failure

of renal function there was a lowered tolerance to BAL, but this was not pronounced

3 Animals with hepatic damage experienced toxic symptoms and some died after doses of BAL well below the fatal level for normal animals

4 It is suggested that care should be exercised when BAL is given to patients suspected of impaired liver function. The presence of severe renal disease does not appear to be a contraindication to BAL.

We are indebted to the Director-General of Scientific Research (Defence) for permission to publish this paper, and to Prof R A Peters, F.R.S., for much assistance

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THE ABSORPTION OF SOME SULPHAGUANIDINE DERIVATIVES IN MICE

BY

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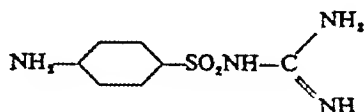
(Received October 17 1946)

Sulphaguanidine (II) was independently prepared by Marshall, Bratton, White, and Litchfield and Roblin, Williams, Winnek, and English in 1940, and introduced for the treatment of bacillary dysentery on the basis of its poor absorption from the gut. It is now well established that orally administered sulphaguanidine attains only low concentrations in the blood of man (Marshall, Bratton, Edwards, and Walker, 1941, Anderson and Cruickshank, 1941, Beling and Abel, 1941, Frisk, 1941) and animals (Marshall, Bratton, White, and Litchfield, 1940, Roblin, Williams, Winnek, and English, 1940, Cameron and McOmie, 1941, Zozaya, 1941, Ambrose and Haag, 1942, Rose and Spinks, 1946), and that this is due partly to the absorption of only some 30–50 per cent of the administered drug and partly to a rather rapid rate of clearance by the kidney (Frisk, 1941, Zozaya, 1941, cf Rose and Spinks, 1946, Fisher, Troast, Waterhouse, and Shannon, 1943). Little light has been thrown on the physical or chemical characteristics responsible for this poor absorption, which might be due either to a slow rate of diffusion across the intestinal membranes or to a partial availability of drug within the gut. Sulphaguanidine is relatively highly soluble compared with, for example, sulphapyridine and sulphathiazole, so that any such partial availability cannot be ascribed to low solubility over the intestinal pH range.

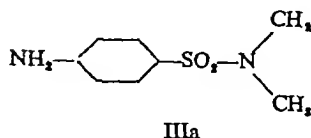
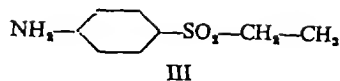
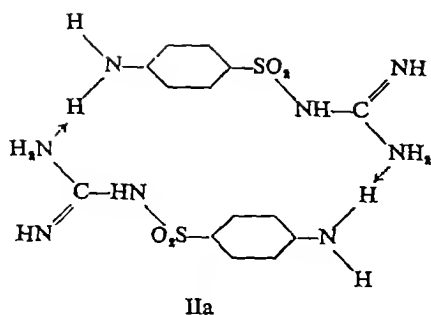
Whatever may be the cause of this phenomenon, it is not immediately apparent how such a striking effect could arise from the simple difference in chemical structure between the parent sulphanilamide, which is very well absorbed, and sulphaguanidine.



I



II



Sulphanilamide (I) is derived from ammonia by replacement of a hydrogen atom of the latter by the sulphanilyl radicle. Although ammonia is a base, that is, in aqueous solution it acquires a proton to form the ammonium ion, the influence of the sulphonyl group is such that in sulphanilamide this tendency is reversed and the sulphonamide group exhibits feeble acidic properties, that is, it tends to liberate a proton. Guanidine is, however, a much stronger base than ammonia, and in combination with the sulphonyl group provides the sulphonyl guanidine group, which is approximately electrochemically neutral, that is, it is neither markedly acidic nor basic. Krebs and Speakman (1946) have suggested as a corollary to their work on solubility and dissociation constants in the sulphanilamide series that poor absorption from the intestine is a result of this suppression of ionization. In our opinion, it is unlikely that this effect, which constitutes the main chemical difference between sulphanilamide and sulphaguanidine, can be the primary cause of their different pharmacological behaviour, otherwise compounds such as *p*-aminophenylethylsulphone (III) and sulphanildimethylamide (IIIa) which carry the non-ionizing sulphonylethane and sulphonyldimethylamide groups, respectively, might also be expected to resemble sulphaguanidine. As is shown below in Tables I and II, both of these compounds are relatively well absorbed, the former to the same extent as sulphanilamide.

We were therefore led to consider the possibility that a physical difference between sulphaguanidine and sulphanilamide other than that suggested by Krebs and Speakman might account for their observed physiological behaviour.

The work of Hunter (1941) has drawn attention to the capacity of amidine and guanidine groups to take part in hydrogen bond formation, and we tentatively supposed that similar effects involving both the sulphonylguanidine group and the primary amino group in the *para* position of the benzene ring might account for the poor absorption of sulphaguanidine through union with some substrate or substrates to be found in the gut contents. A further factor might be the formation of dimeric molecules of type (IIa) in which, it is suggested, hydrogen bonds again play a part. It is presupposed that in either event, the sulphonylguanidine and *p*-amino groups operate simultaneously, the former as a hydrogen

acceptor and the latter as a hydrogen donor. Any charge displacement resulting from the acceptance of a hydrogen atom in one part of the molecule would then be balanced by an opposite and approximately equal displacement in another.

Union with a substrate would necessarily reduce the amount of drug available for absorption, while the increased molecular weight resulting from dimer formation would probably lead to slower absorption. In the latter connection, sulphanilamide derivatives of molecular weight comparable to that of the hypothetical dimer, which have been studied in these laboratories, for example some sulphanilamidoquinolines, have attained only low concentrations in the blood of mice, and it must be emphasized that what is to be explained is not a complete failure of absorption, but partial absorption to a degree not much less, in some species, than that shown by, say, sulphapyridine.

The first hypothesis, that of union with a substrate, cannot easily be tested by direct experimentation. The second should be capable of proof or disproof by molecular weight determinations in aqueous solution. So far, however, it has not been possible to obtain unequivocal results by such determinations. Indirect evidence for an effect involving two point hydrogen bonding has been sought by examining the absorption in mice of a number of sulphaguanidine derivatives. In these the molecule has been modified so that the possibility of association through hydrogen bonding is either left unchanged or is reduced or wholly eliminated. The several chemical types were selected on the following basis:

Type I Compounds carrying substituents in the guanidine residue

A twofold influence was anticipated in this instance. The introduction of one or more alkyl groups on the terminal nitrogen atom would enhance the electronegativity of the latter and might therefore be expected to strengthen hydrogen bond formation. Against that, the steric effect of the substituents, depending upon their sizes, should inhibit the necessary close approach to a second molecule, and would probably be the more important factor.

Type II Compounds in which double hydrogen-bonding is not possible

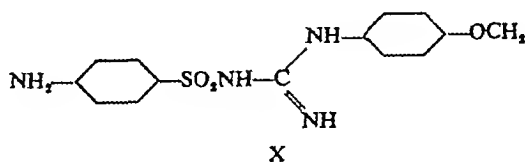
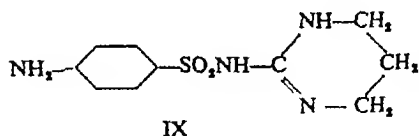
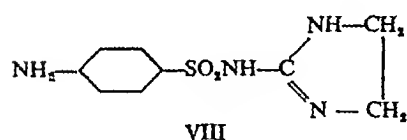
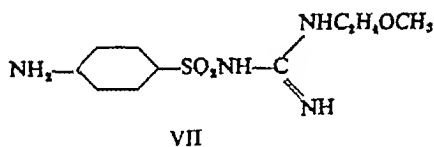
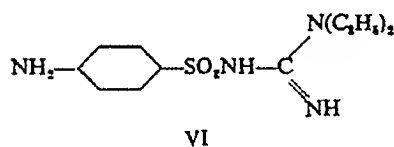
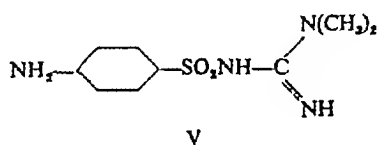
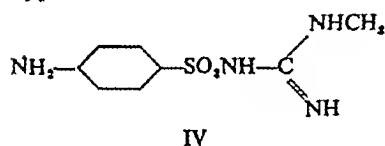
The substitution of nitro for the primary amino group (XI) or the transference of the latter to the *meta* position (XII) would effectively modify the formation of the double hydrogen-bond systems, in the first compound because the nitro group is devoid of the necessary hydrogen atom, and in the second because of the markedly different relative positions of the significant groups in the drug molecule, rendering impossible, for example, the achievement of a structure such as (IIa). A further compound (XIII) was also included under this heading, in which it was anticipated that the presence of a methyl in the *ortho*

position to the primary amino group might inhibit to some extent the participation of the latter in hydrogen bond formation. The correspondingly substituted sulphanilamide (XIIIa) was examined as a control substance.

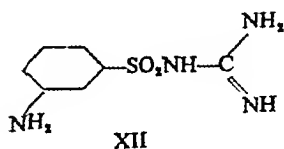
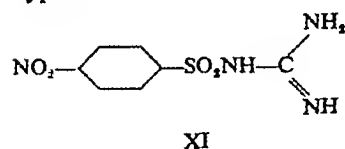
In addition to compounds classified under the above two headings, we included in this investigation several miscellaneous substances such as the two acetyl derivatives of sulphaguanidine (XIV) and (XV), and the sulphonyl acetamide (XVI) which closely resembled sulphaguanidine chemically, and which could be involved in hydrogen bond formation in the same manner as the latter drug.

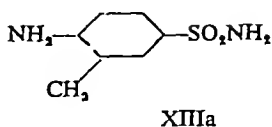
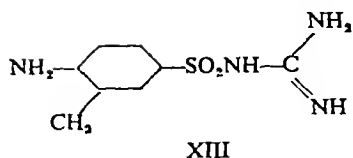
The substances employed in this investigation are listed below. For the preparation of some of them we are indebted to our colleagues, Dr F H Slinger and Dr G Swain. Details of the chemical work will be described in the *Journal of the Chemical Society*.

Type I

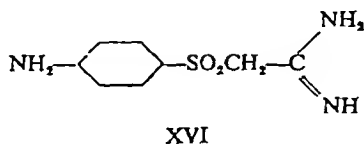
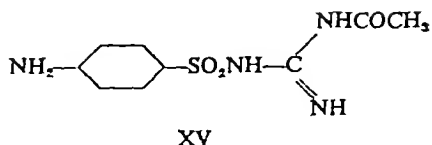
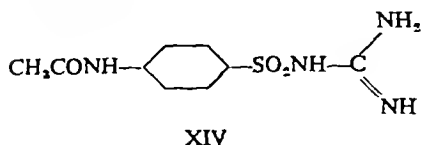


Type II





Miscellaneous



A few compounds of Type I have been described by Winnek, Anderson, Marson, Faith, and Roblin (1942) who also recorded the maximum blood concentrations attained following the oral administration to mice of single doses of 0.5 g/kg. They concluded from experiments with the ethyl, propyl, and butyl homologues of (IV) that the presence of the alkyl groups led to an increased absorption, the increase being particularly marked in the case of the propyl derivative.

EXPERIMENTAL

1 Analytical methods

With two exceptions (XI and XIV) each compound was determined by the micro method of Rose and Bevan (1944). The advantages of this method have recently been summarized elsewhere (Spinks and Tottey, 1946). Adequate recoveries (± 10 per cent) of each compound were established by trial analyses of known amounts added to blood.

p-Nitrobenzenesulphonylguanidine was determined by the following method based on that of Eckert (1943) for *p*-nitrobenzoic acid. 0.02–0.04 ml blood was measured and pipetted into 0.4 ml water in a test tube (18 × 100 mm) and 0.2 ml trichloroacetic acid added. After centrifugation, the clear supernatant fluid was decanted into a similar tube graduated at 2 ml, the protein residue being washed with 0.2 ml water. One drop of Eckert's tartaric acid mixture was then added, followed by 1 drop of *N*/20 titanous sulphate and the tube was heated for 10 min at 100° C. On cooling, 0.2 ml 0.10 per cent sodium nitrate was added and 20 min later, 0.4 ml of 1 per cent *N*- β -sulphatoethyl-*m*-toluidine (Rose and Bevan, 1944). Thirty min were allowed for coupling and the volume was then made up to the mark with distilled water. Immediately before a reading in the colorimeter each tube was centrifuged since some turbidity was usually evident. This procedure was found to give only 70–80 per cent recoveries of *p*-nitrobenzenesulphonylguanidine from

blood Eckert obtained similar results with *p*-nitrobenzoic acid. Instead of using a conversion factor, unknown samples were read against standards (0, 0.01, 0.03, 0.05, 0.07, and 0.10 ml. volumes of a 10 mg/100 ml aqueous solution of *p*-nitrobenzenesulphonylguanidine), to which blood (0.04 ml) had been added. In spite of the centrifugation immediately before colorimetry individual blood concentration-time curves obtained for *p*-nitrobenzene

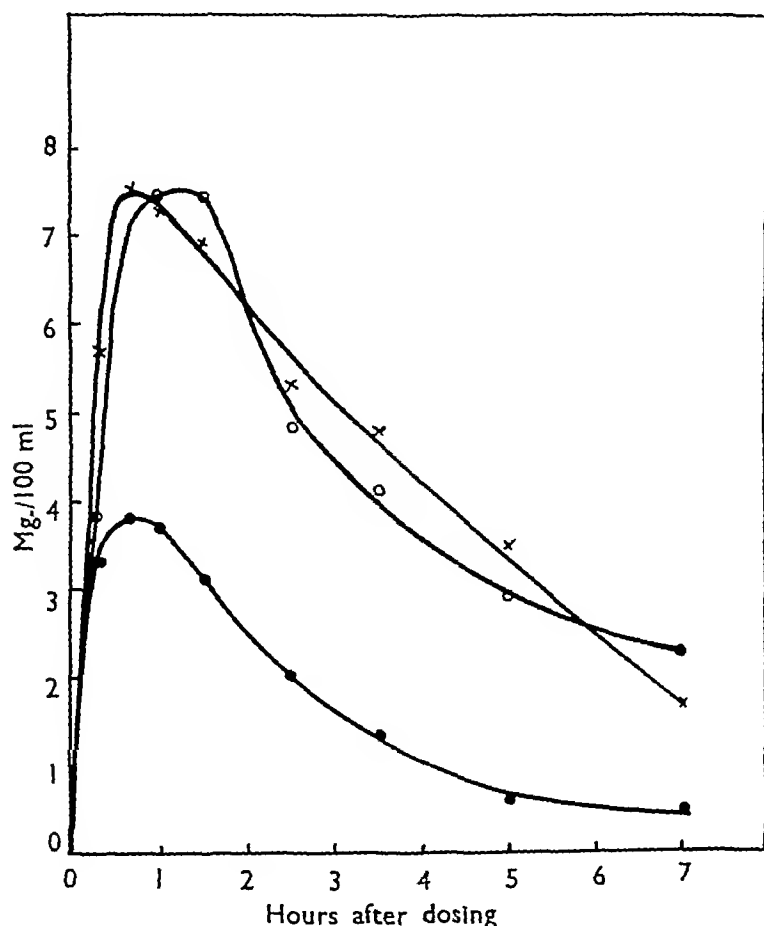


FIG 1—Mean blood concentration-time curves in mice of sulphaguanidine (II, ●—●, 30 animals), metanilylguanidine (XII, ○—○, 18 animals), and *p*-nitrobenzenesulphonylguanidine (XI, ×—×, 24 animals). Each compound was administered by stomach tube in doses of 5 mg/20 g.

sulphonylguanidine were not very smooth, and it is thought that, owing to residual turbidity, the error of the method may have been as high as 20 per cent. However, the mean curve (Fig. 1) was adequately smooth. The final result, of course, included the *p*-nitrobenzenesulphonylguanidine that had been converted to sulphaguanidine *in vivo*. The extent of such

conversion was separately examined, the blood from mice receiving 5 mg of *p*-nitrobenzenesulphonylguanidine per 20 g being analysed for sulphaguanidine itself. No sample contained more than 0.5 mg/100 ml of the latter. *p*-Acetamidobenzenesulphonylguanidine (XIV) was determined by the method of Rose and Bevan (1944) for total amine, modified so that the protein precipitation was carried out at about 1 in 50 dilution instead of 1 in 20. This involved an increase in the final volume of solution to 5 ml, but satisfactory sensitivity was achieved by reading in 2 cm 6 ml cells in the Morris colorimeter.

II Biological methods

The standard conditions used by us in the examination of sulphonamide blood concentrations in mice have already been described (1946) and the original publication should be consulted for full details. Each compound was administered orally to a group of 3 mice in doses of 5 mg./20 g and as a 1 per cent solution (of the hydrochloride) or dispersion, pooled tail blood from the 3 animals being analysed at intervals after dosing.

This individual experiment was repeated at least 6 times for each drug, and characteristic values obtained from the individual smooth curves as already described (Rose and Spinks, 1946), including maximum concentration (max) the time after dosing at which this was attained (*t* max), and the time (*C* 5) taken for the concentration at 5 hours to fall to two-thirds of the value then observed. The results are given in Table I. The mean results are summarized in Table II, in which the compounds have been arranged in order of falling maximum blood concentration. Limits of error of means are for a probability level of 0.05, that is, there is one chance in twenty of the error exceeding the given limit. A value differing significantly ($P=0.05$) from that for sulphaguanidine is printed in *italics*, one differing decisively ($P=0.01$) in **bold type**.

Two compounds only (IV and X) differed significantly from sulphaguanidine in rate of disappearance from the blood. It may therefore be concluded that the maximum blood concentration is a fairly satisfactory index of the extent of absorption from the gut.

TABLE I

BLOOD CONCENTRATIONS OF SULPHAGUANIDINE DERIVATIVES AND ALLIED COMPOUNDS

Compound No	No of experiments	Mean blood concentrations in mg/100 ml after									Values from individual curves		
		20 min	40 min	1 hr	1½ hr	2½ hr	3½ hr	5 hr	7 hr	24 hr	Max (mg./100 ml)	<i>t</i> max (min.)	<i>C</i> 5 (hr)
III	7	7.7	10.3	10.6	10.6	7.2	3.8	1.7	0.9	0.1	11.7	63	1.1
	Standard deviations										3.0	21	0.5
	Limits of error of means										±2.7	±19	±0.5
IIIa	8	6.1	8.0	8.3	7.8	6.5	4.2	3.15	1.2	0	8.9	71	1.2
	Standard deviations										1.6	19	0.6
	Limits of error of means										±1.3	±15	±0.45

TABLE I—continued

Compound No	No of experiments	Mean blood concentrations in mg/100 ml after —										Values from individual curves		
		20 min	40 min	1 hr	1½ hr	2½ hr	3½ hr	5 hr	7 hr	24 hr	Max. (mg / 100 ml.)	t max (min)	C.5 (hr)	
IV	9	2.8	3.8	3.9	3.8	2.7	1.8	1.1	0.8	0.2	3.9	67	13	
	Standard deviations										1.6	8	0.4	
	Limits of error of means										±1.2	±6	±0.3	
V	6	7.8	8.0	7.3	5.5	3.4	2.25	1.4	1.0	0.2	8.35	42	1.4	
	Standard deviations										2.3	24	1.1	
	Limits of error of means										±2.3	±24	±1.1	
VI	6	5.9	7.05	7.4	7.5	6.3	4.65	3.4	1.9	1.0	7.9	98	2.6	
	Standard deviations										1.5	42	1.7	
	Limits of error of means										±1.5	±42	±2.0	
VII	8	3.2	6.0	5.5	4.6	3.2	2.1	1.2	0.95	0.3	5.7	72	1.6	
	Standard deviations										1.45	13	1.0	
	Limits of error of means										±1.2	±11	±0.9	
VIII	7	4.3	6.0	7.3	7.1	5.6	3.6	2.3	1.2	0.2	7.3	85	1.3	
	Standard deviations										2.0	39	0.4	
	Limits of error of means										±1.8	±36	±0.4	
IX	6	3.35	5.3	5.3	5.2	4.3	2.4	1.2	0.8	0.3	5.6	74	1.3	
	Standard deviations										1.3	23	0.6	
	Limits of error of means										±1.3	±23	±0.6	
X	6	4.6	4.7	3.7	3.2	1.35	0.6	0.15	0.1	0	5.1	37	0.6	
	Standard deviations										1.6	4.5	0.2	
	Limits of error of means										±1.8	±5.2	±0.2	

TABLE I—continued

Compound No	No of experiments	Mean blood concentrations in mg/100 ml after —									Values from individual curves		
		20 min.	40 min	1 hr	1½ hr	2½ hr	3½ hr	5 hr	7 hr	24 hr	Max. (mg./100 ml)	t max. (min)	C.5 (hr)
XI	8	57	75	73	69	53	48	3.5	17	0	7.6	53	17
	Standard deviations										1.8	18	1.6
	Limits of error of means										±1.5	±15	±1.3
XII	6	38	71	77	77	48	41	2.9	2.25	0.6	7.8	82	2.0
	Standard deviations										3.2	35	1.2
	Limits of error of means										±3.2	±35	±1.2
XIII	6	2.55	2.9	2.75	2.4	1.6	1.6	0.85	0.5	0.2	3.1	48	1.3
	Standard deviations										0.8	18	0.4
	Limits of error of means										±0.8	±18	±0.4
XIIIa	6	10.5	13.1	13.3	12.9	10.6	6.8	5.0	2.9	0.1	13.5	63	1.4
	Standard deviations										3.8	14	0.4
	Limits of error of means										±3.8	±14	±0.4
XIV	6	11	13	14	14	17	1.55	1.2	0.7	0.4	1.95	127	(3.2*)
	Standard deviations										0.7	51	—
	Limits of error of means										±0.7	±59	—
XV	6	3.6	4.8	4.7	4.2	3.7	2.5	2.0	1.0	0	4.75	67	1.3
	Standard deviations										2.0	16	0.7
	Limits of error of means										±2.0	±16	±0.7
XVI	9	14	21	23	20	16	1.2	0.8	0.5	0.1	2.7	75	1.3
	Standard deviations										1.1	34	0.95
	Limits of error of means										±0.85	±25	±0.7

*Does not differ significantly from 0, i.e., no mean is justifiable.

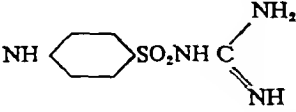
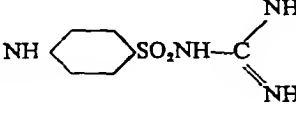
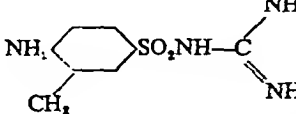
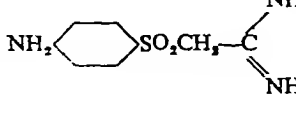
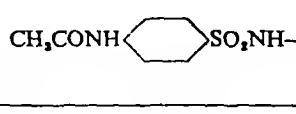
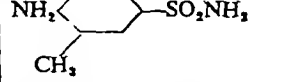


TABLE II

SUMMARY OF MEAN DATA ON THE ABSORPTION OF SULPHAGUANIDINE DERIVATIVES AND ALLIED COMPOUNDS

Figures differing significantly ($P = 0.05$) from those for sulphaguanidine in *italics*, figures differing decisively ($P = 0.01$) in **bold type**.

No	Formula	Max. (mg /100 ml)	t max (minutes)	C.5 (hours)
V		8 35 ± 2 3	42 ± 24	14 ± 11
VI		7 9 ± 1 5	98 ± 42	2 6 ± 2 0
XII		7 8 ± 3 2	82 ± 35	2.0 ± 1 2
XI		7 6 ± 1.5	53 ± 15	17 ± 13
VIII		7 3 ± 1 8	85 ± 36	13 ± 0.4
VII		5 7 ± 1 2	72 ± 11	1 6 ± 0.9
IX		5 6 ± 1 3	74 ± 23	13 ± 0 6
X		5 1 ± 1 8	37 ± 5 2	0 6 ± 0 2
XV		4 75 ± 2 0	67 ± 16	13 ± 0.7

TABLE II—continued

No	Formula	Max. (mg./100 ml)	t max. (minutes)	C.5 (hours)
II		4.3 ± 0.8	50 ± 12	2.2 ± 0.85
IV		3.9 ± 1.2	67 ± 6	1.3 ± 0.3
XIII		3.1 ± 0.76	48 ± 18	1.3 ± 0.4
XVI		2.7 ± 0.85	75 ± 25	1.3 ± 0.6
XVI		1.95 ± 0.7	127 ± 59	—
XIIIa*		13.5 ± 3.8	63 ± 14	1.4 ± 0.4
III*		11.7 ± 2.7	63 ± 19	1.1 ± 0.5
IIIa†		8.9 ± 1.3	71 ± 15	1.2 ± 0.45

* Neither III nor XIIIa differs significantly from sulphanilamide in any respect.

† The significant differences indicated are from sulphaguanidine. The maximum blood concentration is decisively lower than that of sulphanilamide. There is no other significant difference from sulphanilamide.

DISCUSSION OF RESULTS

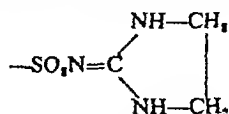
The hypothesis on which this research was based is that sulphaguanidine and related compounds exhibiting poor absorption from the gut owe this property to those features of their molecular structure which permit union with some hypothetical substrate, with the additional possibility of self union to provide a dimeric molecule. It is further supposed that in either event the linking forces are provided by hydrogen bonds associated with the *p*-amino and guanidine groups. The *para* configuration of the sulphaguanidine molecule would be of

special significance in dimer formation, and it is assumed that it might also be of importance in unions of the type postulated with other molecules. These structural features should be reflected in the physical properties of the drug molecules and be capable of measurement. This aspect is receiving the attention of our colleague, Mr J C Gage.

It is convenient to discuss the results obtained under the type headings listed above, noting in each case the extent to which the disturbance of molecular detail has produced the expected effect.

Type I

Of the alkyl derivatives of sulphaguanidine only the monomethyl homologue gives a maximum blood concentration lower than that of the parent compound, and the difference is not significant. The remaining alkyl derivatives all achieve higher maxima in particular the dimethyl and diethyl homologues. The contrast between the former and the monomethyl derivative is marked, and, in so far as hydrogen bond formation is concerned, would indicate that the steric rather than the inductive effect of the alkyl groups is the governing factor, the greater hindrance to the necessary close approach to a substrate or a second molecule being provided, as would be anticipated, by the presence of two alkyl groups. The two ring alkylene compounds (VIII) and (IX) provide a further contrast, the former being absorbed the more completely, although somewhat more slowly. Compound (IX), incidentally, is the tetrahydropyrimidine corresponding to sulphadiazine, a drug which under similar conditions achieves an average maximum blood concentration of 17.5 mg/100 ml (Rose and Spinks, 1946). A theoretical explanation of the difference between (VIII) and (IX) can at this stage be no more than speculation. It is unlikely that the small increase in molecular weight corresponding to the additional methylene group in (IX) is the responsible factor. The main chemical feature of the latter substance would be a tendency for the double bond of the guanidine residue to be stabilized in the heterocyclic ring as formulated, while in (VIII) the disposition of the double bond giving the minimum strain in the ring system might be expected to be that provided in



These differences would almost certainly influence the relative hydrogen-bonding propensities of the ring nitrogen atoms, and be reflected in different degrees of absorption.

Type II

The *meta* isomer of sulphaguanidine (XII) attains a maximum blood concentration which is approximately double that of the parent compound. Some slight change in physical properties (base dissociation constant, solubility, etc.)

would be expected in passing from the one isomer to the other, but it is unlikely that these differences would be solely responsible for the marked increase in absorption of the *meta* compound. At the same time the maximum concentration attained is below the known value for sulphanilamide (13.2 mg /100 ml.) It would appear, therefore, that the sulphonylguanidine grouping is *per se* less favourable to absorption from the gut than the sulphonamide group and that this inhibiting effect is markedly increased in association with a *para* amino group. The close identity of the absorption data of (XII) and the nitro compound (XI) (see Fig. 1) adds further support to these views. In the latter substance a *para* substituent is present, but it is of a chemical type unable to provide hydrogen atoms for bond formation.

The presence of a methyl group *ortho* to the amino group of sulphaguanidine (XIII) suppresses rather than enhances absorption. Reference to formula (IIa) shows that for the methyl group to inhibit hydrogen bonding to any marked extent, the molecules must be oriented with the alkyl groups adjacent to, and on the same side as, each of the two hydrogen bonds. No inhibition would be expected with the methyl groups in the alternative *ortho* positions. The actual experimental results are inconclusive, therefore, as regards substantiation of the hypothesis. It should be noted that compound (XIIIa), which was included as a control substance in this aspect of the investigation, does not differ significantly in its absorption characteristics from sulphanilamide (Rose and Spinks, 1946).

Among the miscellaneous substances included in this research, the results obtained with the sulphonylacetamide (XVI) are of some significance. This compound differs structurally from sulphaguanidine only in that the imido group of the latter is replaced by a methylene linkage. The terminal amidine residue $-C(=NH)-NH_2$ is common to both. Since both compounds are poorly absorbed, it would appear that the amidine moiety is the controlling feature. This is in accord with expectations. On the other hand the experimental findings with the acetyl derivative of sulphaguanidine (XV) were not anticipated. The combined influence of the sulphonyl and acetyl groups on the guanidine residue is such that this substance regains the weak acidic properties characteristic of the simple sulphonamide group. The guanidine group in (XV) would, therefore, be expected to function less readily as a hydrogen acceptor and the compound should then resemble sulphanilamide rather than sulphaguanidine. Against this, it has been observed that the acetyl group of (XV) is removed with unusual ease, for example, on standing for a short period of time in cold dilute sodium hydroxide solution, to regenerate sulphaguanidine, and it may be that such deacetylation occurs in the gastro-intestinal tract resulting in an observed absorption characteristic of the latter drug.

In conclusion it is apparent that the results of the experiments *in vivo* recorded above can at best provide only indirect substantiation of the hypothesis proposed. Further, with compounds such as the alkylguanidine derivatives, two

effects were expected to result from the introduction of the alkyl groups, the one tending to decrease, the other to increase the stability of hydrogen bond formation, but in the absence of the necessary physical data relating to these substances it was not possible to forecast with certainty which feature would predominate. However, the marked contrast between the absorption of sulphaguanidine and, for example, its diethylhomologue (VI), in which the molecular weight is increased by nearly sixty units, would be contrary to expectations were it not postulated that the action of the alkyl groups is one of steric hindrance to the approach to the guanidine residue of some other chemical structure which by its presence would inhibit passage of the drug through the gut wall. The increased absorption noted by Winnek *et al* (1942), in particular of the monopropyl derivative of sulphaguanidine, can also be accounted for on the same basis. The conclusions to be drawn from the data for the *p*-nitro (XI) and *m*-amino (XII) compounds are more precise. Clearly the capacity of these compounds to be involved in association with a second structure in which the potential hydrogen bonding is oriented to accommodate the *p*-aminobenzenesulphonyl guanidine molecule will be markedly influenced in these two instances in favour of more ready absorption from the gut.

SUMMARY

The blood concentrations, following oral administration to mice, of a number of derivatives of sulphaguanidine have been determined, and the results analysed in the light of the hypothesis that the poor absorption from the gut of the parent compound is associated with structural features which may permit this substance to exhibit hydrogen bond phenomena. While much of the experimental evidence circumstantially supports this view, some of the results are inconclusive.

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THE EFFECTS OF PYRIMIDINE SULPHONAMIDE DERIVATIVES UPON THE BLOOD-CLOTTING SYSTEM AND TESTES OF CHICKS AND THE BREEDING CAPACITY OF ADULT FOWLS

BY

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Caecal coccidiosis in chickens can be effectively treated with certain sulphonamides, particularly sulphamezathine (sulphadimethylpyrimidine) (Horton-Smith and Taylor, 1943) and sulphapyrazine (Horton-Smith and Boyland, 1946) Sulphamezathine was extensively used in the treatment of outbreaks of caecal coccidiosis on poultry farms during the 1945 and 1946 seasons On some of these farms a few chicks did not thrive during treatment and a small proportion died The only apparent abnormality found on post-mortem examination of such chicks was the presence of haemorrhages of the intestines and occasionally of the liver and skeletal muscles The appearance of such chicks resembled that described in vitamin K deficiency and the haemorrhagic form of sweet clover disease In order to investigate this syndrome, sulphamezathine was dosed to chicks experimentally and was found to produce hypoprothrombinaemia in most chicks Internal haemorrhages, similar to those found in the field, were observed in a small proportion of cases

In the course of these experiments cockerels dosed with sulphamezathine for periods longer than 10 days showed premature development of combs and wattles, such cockerels had enlarged testes with hypertrophy of the seminiferous tubules A brief account of these effects has been published (Asplin, Boyland, and Horton-Smith, 1946)

EXPERIMENTAL

Except where otherwise stated Rhode Island Red (R.I.R.) chicks of mixed sexes or Light Sussex \times Rhode Island Red cockerels were kept on dry standard mash or placed on experimental diets when one week old The sulphonamides were administered as solutions of the sodium salts in the drinking water In some experiments chicks were weighed at weekly intervals to determine the effect of treatment on body growth Blood from recently killed chicks was taken by heart puncture and placed in tubes containing sodium oxalate The plasma was separated by centrifugation and the prothrombin time determined by a modification of the method of Witts and Hobson (1940), the only change in the method was the omission of lecithin The prothrombin times were determined on undiluted plasma

and on plasma diluted four times with saline, in the presence of Russell viper venom (0.004 per cent (w/v)) and CaCl_2 (0.1 M)

A lengthening of clotting time was observed regularly in chickens dosed with 0.2 per cent (w/v) sulphamezathine, in some cockerels, similarly dosed, there was also enlargement of the combs and wattles as shown in Fig 1. Cockerels with enlarged combs invariably had enlarged testes, which was clearly shown when the individual gonad weights were expressed in mg per 100 g body weight

TABLE I

THE EFFECT OF ADDITION OF VARIOUS SULPHONAMIDES TO THE DRINKING WATER OF GROUPS EACH OF 7 YOUNG CHICKS FOR 28 DAYS

All values are means for the groups

Drug 0.2 per cent (w/v)	Sex	Body weights at end of dosing g.	Haemoglobin g per 100 ml	Prothrombin time (sec.)		Gonad weight (mg.)	
				undil	dil $\times 4$	actual	mg. per 100 g. b wt
Sulphadiazine	M	255	8.9	26.4	47.3	29.7	11.6
	F	282				136.5	48.0
Sulphathiazole	M	321	10.2	26.2	47.2	48.3	15.0
	F	284				133.7	47.1
Sulphamerazine	M	290.2	9.5	27.3	49.6	54.6	18.8
	F	228.8				122.3	53.4
Sulphamezathine	M	224.5	8.9	36.2	81.4	63.1	28.1
	F	265.5				149.5	56.3
Sulphapyrazine	M	293.4	9.3	22.9	43.0	30.9	10.5
	F	315.8				146.8	43.3
Water control	M	275.4	9.8	23.2	41.8	39.4	14.3
	F	289.2				137.2	47.5

Results of a typical experiment in which groups, each of seven 7-day-old R.I.R. chicks, were dosed with different sulphonamides are shown in Table I. Sulphamezathine treatment appears to reduce body growth more than any of the other drugs and has a much greater effect on prothrombin clotting time and testes weight than any of the other compounds tested. None of the compounds used

TABLE II

EFFECT OF ADMINISTRATION OF SULPHAMEZATHINE AND *p*-AMINO BENZOIC ACID IN DRINKING WATER FOR 28 DAYS ON TESTES WEIGHT AND PLASMA PROTHROMBIN OF COCKERELS

Treatment	No of chicks	Prothrombin time sec. (means) undil plasma	Testes weight (mg.) (means)	
			actual	mg. per 100 g body wt
Sulphamezathine 0.2%	6	43.3	51.6	24.2
Sulphamezathine 0.2% + <i>p</i> -aminobenzoic acid 0.02%	6	39.3	77.4	34.4
<i>p</i> -aminobenzoic acid 0.02%	6	26.3	43.8	13.7
Water control	6	26.1	34.7	13.0

in a concentration of 0.2 per cent (w/v) had any marked effect upon the blood haemoglobin concentration or female gonad weight

The therapeutic effect of sulphamezathine or sulphapyrazine on coccidiosis is neutralized by *p*-aminobenzoic acid. The results shown in Table II demonstrate that neither the effect of sulphamezathine on the development of the testes nor on the prothrombin is neutralized by addition of *p*-aminobenzoic acid. The figures for body growth (Table III) show that sulphamezathine reduced the rate of body growth and that normal growth was not restored by addition of *p*-aminobenzoic acid. These results indicate that the effects of sulphamezathine on blood prothrombin and on body growth are not due to a reduction in vitamin synthesis owing to partial sterilization of the gut, although if *p*-aminobenzoic acid is more

TABLE III
AVERAGE WEIGHT IN GRAMS OF CHICKS AT 7-DAY INTERVALS

Treatment	No of chicks	7 days (i.e., initial wt.)	14 days	21 days	28 days	35 days
Sulphamezathine	11	55.0	74.2	113.6	164.0	204.1
Sulphamezathine + <i>p</i> -aminobenzoic acid	12	56.2	80.8	118.3	162.5	211.9
<i>p</i> -aminobenzoic acid	12	54.6	97.7	144.8	226.9	277.6
Water control	12	54.9	97.3	149.0	229.7	275.8

completely absorbed than sulphamezathine the latter might still inhibit vitamin synthesis by intestinal flora, it was, however, found that the administration of sodium succinyl sulphathiazole (0.2 per cent (w/v) of drinking water) had no effect on body growth, prothrombin time, or testes size of chicks

TABLE IV
THE EFFECT OF VITAMIN K ON THE BLOOD PROTHROMBIN LEVELS OF CHICKS TREATED WITH SULPHAMEZATHINE

(a) Groups of 7-day-old chicks under experiment for 17 days

Food	Supplements	No of chicks	Prothrombin time (sec.)	
			undil.	dil $\times 4$
Standard mash	None	8	23.3	39.4
Standard mash	Sulphamezathine (0.2%) in drinking water	8	32.9	55.5
Vitamin K deficient	None	6	53.5	82.2
Vitamin K deficient	Sulphamezathine (0.2%) in drinking water	6	46.1	71.8
Vitamin K deficient	Sulphamezathine (0.2%) in drinking water, menaphthone (10 mg. per kg.) in food	6	19.25	33.2
Vitamin K deficient	Menaphthone (10 mg. per kg.) in food	5	18.2	34.2

TABLE IV—*continued*
 (b) Groups of 8-day-old chicks under experiment for 15 days

Supplement	No of chicks	Final body wt (grams)	Prothrombin time		Testes weight (mg.)	
			undil	dil. $\times 4$	actual	mg./100 g body wt.
Sulphamezathine, 0.2% sol	6	125.5	36.8	54.7	28.4	21.9
Sulphamezathine, 0.2% sol + menaphthone, 10 mg./kg food	7	126.7	29.0	41.1	39.6	30.4
Menaphthone, 10 mg./kg. food	7	135.3	25.4	37.1	15.4	11.3
None	7	140.7	27.9	38.7	16.9	12.0

(c) Groups of 9-week-old cockerels under experiment for 26 days

Supplement	No of chicks	Increase in wt during expt (g.)	Prothrombin time (sec.)	
			undil	dil. $\times 4$
Sulphamezathine, 0.2% sol	10	353.6	71.1	124.6
Sulphamezathine, 0.2% sol + synkavit 0.0025%	10	262.6	30.7	51.2
Sulphamezathine 0.3% + synkavit 0.0025%	10	257.9	30.1	56.6
None	6	388.7	33.1	60.8

The effect of sulphamezathine on blood prothrombin is similar to but not so great as that produced by vitamin K deficiency, and it was completely neutralized by the addition of menaphthone (vitamin K) to the diet (Table IVa), the effect of sulphamezathine on testes development, however, was not influenced by vitamin K (Table IVb). The effect of combining soluble vitamin K (calcium-2-methyl-1,4-dihydroxynaphthalenediphosphoric ether, "synkavit" Roche) and sulphamezathine in a single solution gave similar results (Table IVc). However, in a further experiment (Table VI, expt E) three of a group of seven older chickens which received sulphamezathine (0.25 g per kg body weight daily), combined with vitamin K (menaphthone, 25 mg per kg body weight), showed intestinal haemorrhages on post-mortem examination, this indicates that haemorrhagic lesions in older birds may not be entirely due to deficiency of prothrombin.

Sulphamezathine has a rapid effect upon the blood clotting mechanism similar to that of dicoumarin (3,3'-methylenebis-4-hydroxycoumarin), the causative agent of sweet clover disease in cattle. Sulphamezathine appears to be quantitatively less effective than dicoumarin (Table V) because a single dose of 2 g per kg body weight sulphamezathine had less effect on prothrombin time than 150 mg per kg dicoumarin. The effect of sulphamezathine on prothrombin level disappears between four and ten days after the withdrawal of sulphamezathine.

Although the effect of sulphamezathine on blood clotting develops rapidly, the addition of varying amounts of sodium sulphamezathine to plasma *in vitro* was found to have no effect on the prothrombin time. Sulphamezathine had no

TABLE V

THE EFFECT OF LARGE DOSES OF SULPHAMEZATHINE AND DICOUMARIN ON PLASMA PROTHROMBIN OF 23-DAY-OLD CHICKS

Prothrombin times (sec.)

Sulphamezathine, 3 doses of 1 g./kg body wt. 42, 26 and 18 hr before sampling		Sulphamezathine, single dose of 2 g./kg. body wt. 18 hr before sampling		Dicoumarin, 150 mg per kg body wt 18 hr before sampling		Normal control	
undil	dil \times 4	undil	dil \times 4	undil	dil \times 4	undil	dil \times 4
24.2	61.0	39.4	68.8	48.0	113.8	19.2	44.6
27.2	69.4	44.2	110.4	33.8	78.4	24.4	49.6
26.4	58.2	46.8	87.6	54.4	112.2	25.0	36.4
28.6	58.4	29.0	61.4	43.0	118.2	30.4	58.8
		30.8	69.0			25.2	42.0
Mean 26.6	61.75	38.0	79.4	44.8	105.7	24.8	46.3

direct anticoagulant action similar to that of heparin or of dyes such as chlorazol fast pink, the effect on blood clotting *in vivo* may therefore be independent of the actual concentration of the drug at the time when the blood is taken

Haemorrhagic lesions in chicks

A proportion of chicks dosed with sulphamezathine have developed multiple haemorrhages, while no such haemorrhages have been seen in a large number of control chicks, or in chicks treated with other sulphonamides. It is therefore clear that this syndrome is induced by sulphamezathine, and the experiments described suggest that the action is similar to that of sweet clover in producing haemorrhagic disease in cattle. In various experiments a total of ninety-one 7-day-old chicks were given a 0.2 per cent (w/v) solution of sulphamezathine for periods of 21–35 days, intestinal haemorrhages were present in 6 cases only and there were no deaths attributable to haemorrhage. In all chicks of this group lesions were confined to the intestines, they varied from very numerous petechiae extending throughout the greater part of the length of the intestine to relatively few larger haemorrhages of 3–5 mm diameter. The haemorrhages were readily visible through the serosa. The lesions occurred in all layers of the bowel wall, but were most frequent in the submucosa.

Chickens treated for caecal coccidiosis on farms are usually between 6 and 12 weeks old. All field cases of sulphamezathine poisoning, characterized by haemorrhagic lesions, which have been brought to our attention have occurred in chickens of this age.

The incidence of haemorrhagic lesions shown in Table VI indicates that chickens of 6 weeks old are more susceptible to the haemorrhagic effect of sulphamezathine than are younger chicks. For instance, in experiment E, 7 out of 13 chickens killed after 3 weeks' treatment with 0.25 g sulphamezathine per kg.

TABLE VI

THE EFFECT OF ADDITION OF SULPHAMEZATHINE TO DRINKING WATER OF 6-12-WEEK-OLD CHICKENS

Exper	No of chickens	Age	Dose	Period of treatment	Effect
A	8	7 days	0.2% sol	100 days	Two died from haemorrhage on 44th and 50th days. Six survived and remained healthy.
B	9	9 weeks	0.2% sol	97 days	None died. Precocious development of comb and wattles and male plumage.
C	11	6 weeks	0.2% sol	62 days	Four died from haemorrhage on 18th, 36th and 39th days, two killed on 63rd day showed small intestinal haemorrhages. Five normal.
D	15	6 weeks	0.2% sol	70 days	Three died, 26th, 44th and 56th days, with haemorrhages. Four killed showed haemorrhages. Eight normal.
E	7	6 weeks	0.25 g per kg body wt daily	20 days	None died. Four showed haemorrhages when killed on 21st day.
	6	6 weeks	0.25 g per kg body wt daily + menaphthone 25 mg per kg body wt daily	20 days	None died. Three showed internal haemorrhages when killed on 21st day.
F	7	10 weeks	0.2% sol	15 days	No deaths or gross lesions.
	6	10 weeks	0.2% sol + synkavit 50 mg per l	15 days	No deaths or gross lesions.

body weight daily were found to possess intestinal haemorrhages. Furthermore, 9 out of the 43 chickens receiving 0.2 per cent (w/v) sulphamezathine for 62-100 days (experiments A, B, C, and D) died as the result of internal haemorrhage. Most of the chickens remained in apparently good health after prolonged administration of sulphamezathine, but post-mortem examination of them frequently revealed intestinal haemorrhages similar to those of younger chicks. The lesions in chickens dying as a result of haemorrhage were widespread and severe. Haemorrhages were invariably present in the intestinal tract, the liver also was frequently bespattered with haemorrhagic foci. Massive haemorrhages were seen in the skeletal muscles, particularly in the muscle groups of the thighs and breasts. The ventral border of the gizzard was the site of haemorrhage in two fowls. Persistent bleeding from feather follicles resulted in the deaths of two chicks. It appeared that haemorrhages might occur at any site, but were most frequent at those points particularly exposed to trauma.

Testicular hyperplasia

Precocious sexual development of cockerels was often evident after 10–14 days' dosing with sulphamezathine. With continued dosing, growth of combs and wattles proceeded rapidly, so that after 4 weeks a stage of development normally seen in cockerels of 2–3 months old was reached.

The development of secondary sexual characters was evident in only a proportion of the dosed cockerels. Macroscopic enlargement of testes was invariably present in chicks showing obvious comb and wattle development. Of the 71 chicks receiving sulphamezathine for 21–28 days, 43 had enlarged testes, 21 were approximately normal in size, and 7 were abnormally small. The testes weights of some sulphamezathine-treated chicks after 4 weeks were as much as five times greater than those of untreated control chicks. Sulphamezathine regularly inhibited the body growth of chicks (Tables I and III) and in general it was the chicks which had the best growth rates which developed testicular hyperplasia. A proportion of treated chicks grew very poorly, and in these the testes were very small and inactive.

Sulphamezathine-treated chicks which responded with testicular hyperplasia had haemoglobin and prothrombin levels not significantly different from those of chicks failing to respond in this way (Table VII).

Quantitative data on the enlargement of the testes of cockerels has been given. Table I shows that there was no comparable enlargement of the female gonad after dosing for 4 weeks with any of the sulphonamides tested, and microscopic examination revealed no abnormalities. Sulphamezathine has more effect on the testes than either sulphamerazine (sulphamethylpyrimidine) or sulphadiazine (sulphapyrimidine). Although the average testes weights of chicks dosed with sulphadiazine (Table I) do not indicate enlargement, microscopic examination revealed that the testes of some treated chicks were actively stimulated. Sulphathiazole, succinyl sulphathiazole, and sulphapyrazine had no such action.

The effect was not neutralized by simultaneous dosing of an amount of *p*-aminobenzoic acid which completely neutralized the therapeutic action of the drug in caecal coccidiosis (Horton-Smith and Boyland, 1946, cf. Table II), nor was it neutralized by menaphthone (Table IVb).

Microscopical examination of sections of the cockerel testes showed that the main action of the drug was on the seminiferous tubules which were hyperplastic (Figs 2 and 3). The interstitial tissue remained scanty and appeared to be compressed by the proliferating tubules, but active interstitial tissue was present and this was the probable source of the male hormone. The enlarged seminiferous tubules were lined with spermatocytes in active mitotic division. In the lumen of the tubules there were degenerative forms so that the centre of the tubules sometimes contained a "necrotic area". Sometimes (as in Fig. 4) the hyperplasia occurred in a few tubules, whilst the other tubules remained almost normal and immature.

TABLE VII

A COMPARISON OF THE BODY WEIGHTS, PROTHROMBIN TIMES AND HAEMOGLOBIN LEVELS OF COCKEREL CHICKS WHICH RESPONDED TO SULPHAMEZATHINE DOSING BY PRECOCIOUS SEXUAL DEVELOPMENT, AND THOSE FAILING TO DO SO

Group of cockerels	Body weight (grams)	Testes weight mg /100 g. body weight	Prothrombin clotting times		Haemoglobin g. per 100 ml
			undil	dil \times 4	
Dosed sulphamezathine Good body growth Marked comb development	295	39.6	51.2	73.4	7.84
Dosed sulphamezathine Poor growth No comb development	166	8.5	48.7	68.6	7.54
Control—undosed	308	13.6	34.8	49.6	7.58

The stimulus to sexual development provided by sulphamezathine to cockerels disappeared upon withdrawal of the drug, so that it became impossible to distinguish between dosed and undosed cockerels 3 weeks after the end of the treatment.

Extracts of testes from normal and sulphamezathine-treated cockerels appeared to have no hyaluronidase activity. This is perhaps to be expected, as the immature testes contained no mature spermatozoa.

Microscopic examination of the thyroid glands of the sulphamezathine-dosed chicks failed to reveal any change.

The effects of certain dimethylpyrimidines on cockerel chicks

Only those sulphonamides which contain a pyrimidine ring, i.e., sulphamezathine, sulphamerazine, and sulphadiazine, appear to have any action on the testes. Sulphamezathine, which contains a dimethylpyrimidine ring, was much more active in this respect than either sulphamerazine or sulphadiazine. Groups of 7-day-old chicks were dosed with 0.2 per cent (w/v) solutions of 2-hydroxy-4,6-dimethylpyrimidine (white form), 2-hydroxy-4,6-dimethylpyrimidine (yellow form), 2-amino-4,6-dimethylpyrimidine, and 2-mercapto-4,6-dimethylpyrimidine in place of drinking water. None of these substances had any effect on testes size or prothrombin time. The mercapto compound reduced the rate of growth, but the hydroxy compounds and the amino derivative had no effect on body growth.

The effect of sulphamezathine on drakelets

A group of ten 8-day-old drakelets was dosed with 0.2 per cent (w/v) sulphamezathine in their drinking water for 28 days. Dry food of the same composition as used for chicks was made into a damp mash with sulphamezathine solution. The sulphamezathine was readily consumed. After 28 days' treatment all drakelets were killed and examined. There was no evidence of intestinal haemorrhages in any of the birds. The treatment reduced

the growth rate, but had no effect on testes weight or on prothrombin and haemoglobin levels. The sulphonamide level of the blood of ducklings dosed with 0.2 per cent (w/v) sulphamezathine (4.4 mg per 100 ml) was lower than that of chicks receiving the same dose.

The effect of sulphamezathine on the sex organs of male rats

Groups of ten rats weighing 50–60 g were placed on diets containing sulphonamide (as 0.1 per cent of the dry matter of the diet). Half the rats in each group were castrated just before being put on the diets. The rats were weighed at the beginning of the experiment and at intervals throughout the period of dosing. The data (Table VIII) show that growth was considerably inhibited by sulphapyrazine—rats on this drug hardly grew at all during the last two weeks of treatment—while sulphathiazole had no, and sulphamezathine only a slight, inhibitory effect on growth.

TABLE VIII

THE EFFECT OF DOSING SULPHONAMIDES FOR 34 DAYS ON SEX ORGANS OF MALE RATS

Nature of animals	Mean body wt, g		Testes, mg mean	Seminal vesicles and prostate, mg mean
	Beginning	End		
Undosed normal rats	64	143	850	272
Undosed castrated rats	66	146	—	90
Normal rats dosed with sulphamezathine	55	119	880	426
Castrated rats dosed with sulphamezathine	63	127	—	88
Normal rats dosed with sulphapyrazine	53	71	480	79
Castrated rats dosed with sulphapyrazine	58	86	—	90
Normal rats dosed with sulphathiazole	56	139	960	292
Castrated rats dosed with sulphathiazole	59	135	—	85

None of the drugs produced any marked enlargement of the testes of normal rats, or enlargement of the seminal vesicles and prostate of castrated rats. Sulphamezathine thus has no male hormone action on adult rats and does not cause testicular hypertrophy. On the other hand, the seminal vesicles and prostate of normal rats treated with sulphamezathine were larger, and those of rats treated with sulphapyrazine smaller, than the same organs of rats fed on normal diet or on a diet containing sulphathiazole. Thus although sulphamezathine had no effect on castrated rats and very little on the weight of testes of normal rats, it caused enlargement of the seminal vesicles and prostate presumably by stimulation of the testes.

When rats were dosed with sulphamezathine from birth, hyperplasia of the testes occurred which was obvious in rats weighing less than 50 g. Pregnant rats were fed on a diet containing 0.1 per cent sulphamezathine and the diet was given to the mother and young after birth. The weights of the testes of young rats of different ages dosed with sulphamezathine are compared with the weights of normal animals in Table IX. While the testes weight per 100 g. body weight increases from 380 to 700 mg in normal rats from 16 to 52 g. in weight, the weights vary from 600 to 1300 mg. per 100 g. body weight in sulphamezathine-dosed rats from 17 to 54 g. body weight. Increases in size of seminal vesicles and prostate occurred in young rats, but these were less than the increases in size of these organs in adult rats dosed with sulphamezathine. The increase in testicle size (Figs 5 and 6) is similar to the effect on the cockerel testes.

TABLE IX

THE WEIGHT OF SEX ORGANS OF YOUNG NORMAL MALE RATS AND YOUNG RATS DOSED WITH SULPHAMEZATHINE FROM BIRTH

A NORMAL RATS

Wt of rat, g	16	22	29(2)	32(4)	46(2)	52(2)
Wt of testes, mg	61	97	120	163	303	363
Wt of seminal vesicles and prostate, mg	30	32	47	55	66	71
Testes wt as mg/100 g body wt	380	440	410	510	660	700
Seminal vesicle and prostate wt. as mg/100 g body wt.	187	146	162	172	144	196

B RATS DOSED WITH SULPHAMEZATHINE

Wt of rats, g	17	29(2)	35	38	47(2)	50(2)	54
Wt of testes, mg	103	221	302	330	609	585	702
Wt of seminal vesicles and prostate, mg.	34	48	65	99	94	104	93
Testes wt as mg/100 g body wt	600	760	860	870	1290	1170	1300
Seminal vesicle and prostate wt as mg/100 g body wt.	200	165	186	260	200	204	173

Some figures are averages of groups of rats of the same weight. The number in such groups is indicated in parentheses

The toxicity of sulphamezathine in adult fowls

Two groups of six mature R.I.R pullets and cockerels were dosed by mouth with a 16 per cent (w/v) solution of sodium sulphamezathine at a rate of 0.25 g. per kg body weight daily for 14 days. The pullets which had been in full production ceased to lay on the 3rd day. The fowls became sick and deaths of pullets occurred on the 6th, 7th, 9th, and 16th days, and of cockerels on the 7th, 12th, 13th, 15th, and 16th days. The surviving cockerel remained apparently normal and when killed on the 21st day showed no apparent lesions. The two surviving pullets were very sick on the 15th day but improved by the 21st day, when they were killed. Both had low haemoglobin concentrations (7.5 and 8.1 g. per 100 ml). On post mortem examination both were found to have retained fully formed eggs in the oviduct, the eggs were coated with a fibrinous deposit and appeared to have remained stationary for some time.

Obvious symptoms were only apparent during the four days prior to death although appetite was decreased, the crop remained distended with food, the feathers around the vent were matted and stained with yellow faecal material, the excreta of some birds had a bright greenish-yellow colour which resembled that of fowls affected with fowl typhoid. Some fowls were disinclined to move about and locomotor ataxia was apparent for about 24 hours before death. The colour of the comb and wattles changed in some instances to a bright salmon-red, in one fowl the head appendages became blue and shrivelled. Body temperatures were elevated up to a maximum of 110.4° F. On examination of the cloacal mucosa petechiae could sometimes be observed. In cockerels the genital eminences became green or brown as a result of extravasated blood.

Post-mortem examination revealed widespread lesions. Haemorrhages were invariably present in the intestinal tract, extending from the duodenum to the cloaca (Fig. 7), the gizzard lining of one fowl also was involved. The haemorrhages were similar to those seen in younger chickens and varied in size from petechiae to plaques of 5 mm diameter. The lesions in the large intestine became ulcerated as a result of erosion of the mucosa; the contents of the intestines were mucoid and sometimes bloodstained, petechiae were present in the serous membranes, in the abdominal fat, at the base of the heart, and of the gizzard. The liver was somewhat enlarged and friable, large wedge-shaped infarcts were seen, the

colour of the liver of two fowls was changed to green. The kidneys were enlarged and the colour changed as in the liver. Numerous basophilic erythrocytes appeared before death and the haemoglobin level fell sharply. The spleen was slightly enlarged in some cases and contained subcapsular haemorrhages. The ovarian follicles were soft and flabby, and in one case yolk material was present in the peritoneal cavity. A coliform organism was isolated in pure culture from the spleens of three fowls and heart blood of one. It is probable that the infecting organisms invaded the blood from the gut through the intestinal lesions. Similar effects on the gut were produced when sulphamezathine (250 mg/kg body weight per day) was administered by feeding in capsules (cf Fig 8).

The effect of sulphamezathine on the breeding capacity of fowls

Four 10-month-old R I R cockerels were dosed with 0.15 g. per kg body weight daily. Examination of semen collected from these cockerels before dosing commenced and at intervals during treatment, failed to reveal any change in the yield of semen, or the density, form or motility of sperms. One cockerel died after the 36th dose with lesions of sulpha-



FIG 1—35-day-old cockerels. The two on the left were dosed with sulphamezathine for the last 28 days and show enlarged combs and wattles as compared with the two controls on the right.

mezathine poisoning. After the 50th dose the cockerels were mated to three pens (A, B and C) each of six virgin pullets. The cockerel in pen A died on the 58th day of dosing from sulphamezathine poisoning. Fourteen eggs laid by pullets in pen A between the 53rd and 58th day were incubated. 5 were fertile and 3 hatched normal chicks. Dosing of the cockerels in pens B and C continued until 102 doses had been given but had no apparent effect upon fertility.

A group of six laying hens and a cockerel were dosed with 0.2 per cent (w/v) sulphamezathine in their drinking water. Egg production ceased by the 3rd day, 2 eggs were laid on the 10th day, all six hens had resumed laying by the 15th day. During the succeeding 6 weeks 42 eggs were incubated of which 5 were infertile. 28 normal chicks were hatched from the remaining 37 fertile eggs. Thus the dosing had only a temporary effect on the egg-laying of fowls.

DISCUSSION

As sulphamezathine and sulphapyrazine are so useful in treatment of coccidiosis in chickens it is of value to know what other effects these drugs may have



FIG 2—Section of testis from normal 35-day old cockerel H and E $\times 660$

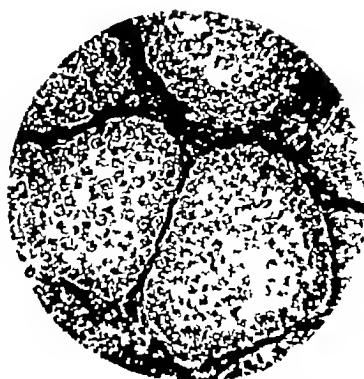


FIG 3—Section of testis from 35-day old cockerel, dosed with sulphamezathine (0.2% in drinking water) for 28 days, showing hypertrophy of tubules H and E $\times 660$



FIG 4—Section of testis from 35-day old cockerel dosed with sulphamezathine showing hypertrophy of part of the tubular tissue while other tubules are not enlarged H and E $\times 100$

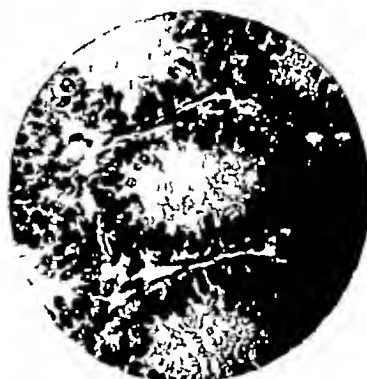


FIG 5—Section of normal testis from rat weighing 16g H and E $\times 660$

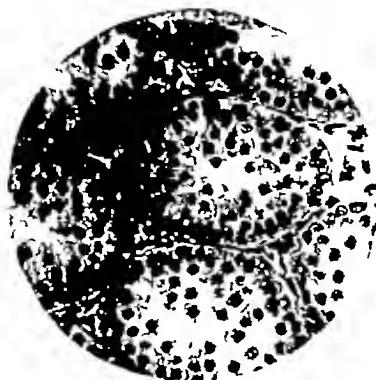


FIG 6—Section of testis from a rat weighing 17 g and dosed with sulphamezathine from birth H and E $\times 660$

While sulphamezathine is liable to cause hyperplasia of the testes of young cockerels, lengthens the blood clotting time, reduces the rate of growth, and causes haemorrhage in the gut, sulphapyrazine has not been found to have any of these effects. Sulphapyrazine is also quantitatively more effective than sulphamezathine in curing coccidiosis, but it is not prepared and is not available in England. In spite of the pathological actions which sulphamezathine can have it remains a valuable drug because the toxic effects are not produced unless treatment is carried on for longer than the recommended period of 7 days. Prolonged treatment has, however, been used by some farmers and a few deaths from haemorrhage have occurred.



FIG 7—Alimentary tract from chicken dosed with sulphamezathine showing widespread haemorrhagic lesions



FIG 8—(a) Duodenum from chicken dosed with sulphamezathine (b) Corresponding duodenum from normal chicken (c) Rectum and caecum from chick treated with sulphamezathine (d) Gizzard and proventriculus from chicken treated with sulphamezathine (e) Omental fat with haemorrhages from chicken treated with sulphamezathine

The increase in therapeutic effect paralleled by an increase in effects on the blood clotting system and on the testes, which occurs when one (sulphamerazine) or two (sulphamezathine) methyl groups are introduced into the sulphadiazine molecule may be due in part to the increased absorption of these methyl sulphapyrimidines.

The effect on blood clotting, and probably to some extent on induction of haemorrhages, can be neutralized by feeding menaphthone or by the addition of a soluble vitamin K preparation to the sulphamezathine solution. In view of this it might be advisable to incorporate a small amount of a vitamin K preparation in sulphamezathine solutions issued for treatment of caecal coccidiosis.

SUMMARY

1 Sulphamezathine, which is an excellent drug for the treatment of caecal coccidiosis in chickens, has certain undesirable side effects. These effects include (a) a decrease in the rate of clotting of the blood, similar to that produced by dicoumarin, which is neutralized by vitamin K, (b) induction of haemorrhages, particularly in chicks between 6 and 12 weeks old dosed for several weeks, (c) a decrease in rate of growth, and (d) a hyperplastic action on the testes of young cockerels, accompanied by growth of the comb and wattles and precocious sexual development.

2 Sulphapyrazine, which is somewhat more effective than sulphamezathine in treatment of coccidiosis, has none of these effects.

3 Sulphamezathine causes hyperplasia of the testes of young rats, but not of adult rats. The increase in testes size is accompanied by some increase of the seminal vesicles and prostate. The drug has no effect on the seminal vesicles and prostate of castrated rats.

4 Sulphamezathine does not appear to affect the fertility of adult cockerels or the laying capacity of fowls.

We wish to thank Dr P. Koller for preparing the microphotographs, Mr D. M. Brown and Dr F. Rose for gifts of the dimethylpyrimidine derivatives, and Dr F. Bergel, of Messrs Roche Products, for synkavit.

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THE COMPARISON OF RESPIRATORY STIMULANT DRUGS

BY

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Drugs stimulating respiration are usually classified as analeptics or as respiratory analeptics. This latter term is perhaps more useful since the term "analeptic" has such a wide significance that, in addition to picrotoxin and leptazol, it can also include such substances as amphetamine and ephedrine, which have an action at higher levels in the central nervous system. The method about to be described has been devised primarily for the more purely respiratory type of analeptic.

The methods usually employed to compare analeptics (using the term generally) are based upon the reduction they produce in the duration of anaesthesia. Trevan (1939) used this principle to compare the central stimulant actions of amphetamine and isomers of ephedrine in mice which had been anaesthetized with paraldehyde. He also included some experiments in which picrotoxin and leptazol were employed as the stimulant drugs, which showed that amphetamine had a greater awakening effect upon mice than either picrotoxin or leptazol, but that leptazol is slightly more effective than picrotoxin.

This type of method is very suitable for the examination of compounds of the amphetamine type in which Trevan was primarily interested, but has several disadvantages when employed to test the more predominantly respiratory stimulants.

Chakravati (1939) pointed out some of the objections to this method and attempted to overcome them. One of the main objections is the variable depth of anaesthesia resulting from the administration of a fixed dose of anaesthetic to a series of mice, and Chakravati attempted to sort out his animals according to the depth of anaesthesia. Despite this, several objections still remain. Firstly, the animals are not necessarily anaesthetized to a constant depth and, secondly, the degree of anaesthesia is not comparable with that occurring in patients when analeptics are required. When these drugs are used clinically it is usually to combat excessive degrees of anaesthesia resulting, most often, from barbiturate anaesthetics.

The criterion by which stimulant drugs will be judged in such clinical use is not so much the extent to which they will awaken the patient as the reliability with which respiration can be improved with their aid. The two effects are inter-related since the awakening effect will follow the use of a successful respiratory stimulant more quickly than if it were not employed.

A most interesting contribution was made to the study of analeptic drugs of this type by Das (1939), who gave continuous intravenous injections of anaesthetics to depress the respiration of rabbits by about 50 per cent and then observed the effects of different stimulant drugs upon that level of anaesthesia. The method we have used is similar to that of Das except that a much more critical level of anaesthesia is employed.

EXPERIMENTAL PROCEDURE

Guinea-pigs weighing between 250 and 700 g. are used, in groups of eight or ten animals, the weight distribution in any set of animals being not more than ± 20 per cent different from the mean value for the group. The animal is secured prone on an operating table and the hair is removed from the outer surface of the forelimbs between the ankle and the knee. A bleb is raised on each forelimb by the injection of 0.1 c.c. of a solution of 2 per cent (w/v) procaine subcutaneously just over the route of the accessory cephalic vein. After a minute the skin in this region is plucked up with forceps and a small patch cut out with scissors, thus exposing about $\frac{1}{4}$ in. of the vein in each forelimb.

The anaesthetic is made up in such a strength that it will kill the guinea pig when infused continuously in about ten minutes. The anaesthetics we have used are thiopentone soluble and pentobarbital soluble, both made up in solutions of 7.5 mg./c.c. The rate of continuous injection employed does not seem very important, but for these solutions 0.6 c.c./min. has been used and found satisfactory.

The anaesthetic solution is injected into the animal by means of a fine dental needle inserted in the vein and attached to a motor-driven syringe containing the solution. With a little practice this injection becomes a simple matter. As injection proceeds the depth of anaesthesia increases until there is considerable respiratory depression. The intervals between successive breaths become longer and longer. When twenty seconds have elapsed after a breath the injection is stopped. At this level of anaesthesia spontaneous recovery is very improbable. If the animal is kept warm, however, the heart will continue to beat for several minutes. The analeptic drug is next injected into the vein of the opposite limb in a similar manner, as a solution containing a half to one LD₅₀ of the substance per c.c. In our experiments a rate of 0.6 c.c./min. was employed.

A true respiratory analeptic will cause respiration to start again in a few minutes and the amount of drug required to do this is recorded. The injection of the drug is continued until there are signs of over-stimulation, such as convulsive jerks, the injection is then stopped and the total amount of the analeptic drug given is recorded.

The guinea-pig is now placed, with the cuts on the forelimbs protected by pads of absorbent cotton wool, in a constant temperature cabinet at 30°C and observed, until it is clear whether the animal will live or die. If the animal lives for two hours death later is very unlikely, animals surviving this period are then destroyed.

We have termed the dose required to restart respiration, and the dose required to produce over-stimulation, *A* and *B* respectively. The values of the *A* and *B* doses per unit weight can be compared for different analeptics.

RESULTS

Experiments were first made to determine for how long the injection of the anaesthetic must be continued after the cessation of respiration in order to avoid spontaneous recovery. The results obtained are given in Table I and indicate

TABLE I

THE EFFECT OF CONTINUING THE INJECTION OF PENTOBARBITAL SOLUBLE (4.5 MG /MIN) AFTER THE LAST BREATH UPON THE MORTALITY OF GUINEA-PIGS

Time lapse after the last breath in seconds	Mortality after 1 hour
0	1/10
5	2/10
10	8/10
20	10/10
20	10/10
20	10/10

that such recovery is very unlikely when twenty seconds have elapsed after the last breath. Table II shows the mean doses of the two anaesthetics required to cause respiratory arrest in seven groups of ten guinea-pigs for each anaesthetic, together with the standard errors of these mean values. The two final means

TABLE II

MEAN DOSES OF ANAESTHETIC REQUIRED TO CAUSE PERMANENT RESPIRATORY ARREST IN GROUPS OF TEN GUINEA-PIGS. BOTH ANAESTHETICS WERE INJECTED AT 4.5 MG PER MINUTE INTRAVENOUSLY

Thiopentone soluble			Pentobarbital soluble		
Group of 10 animals	Mean dose mg./kg.	Standard error	Group of 10 animals	Mean dose mg./kg.	Standard error
1	46.0	1.8	8	68.6	6.0
2	46.7	2.9	9	63.3	4.6
3	44.9	2.5	10	73.6	4.8
4	41.0	3.8	11	68.0	3.7
5	45.2	0.2	12	69.1	2.1
6	50.4	3.2	13	65.5	2.9
7	36.9	1.5	14	64.4	1.7
Mean	44.44	1.04	Mean	67.50	1.6

give an estimate of the LD₅₀ of thiopentone soluble and pentobarbital soluble in guinea-pigs. Each group of ten guinea-pigs was treated on the same day, but the fourteen groups were used over a period of several weeks. It will be seen that the groups of animals give consistent results one with another and the standard errors within each group are small.

For the assessment of a respiratory stimulant drug two groups of ten animals were used, one with each of the anaesthetics, which were chosen to give both short and long durations of anaesthesia, thiopentone soluble was chosen for short and pentobarbital for long durations. The former drug would be more readily antagonized and the latter, owing to its longer action, would provide a better test of the analeptic property.

The results obtained with picrotoxin are given *in extenso* in Table III and the corresponding mean results for leptazol and triazol 156 in Table IV. Triazol

TABLE III

TYPICAL SET OF RESULTS OBTAINED BY THE METHOD DESCRIBED SHOWING THE RELATIVE RESPIRATORY STIMULANT ACTIONS OF PICTROTOXIN UPON THE RESPIRATION DEPRESSED WITH THIOPENTONE AND PENTOBARBITAL. *A* IS THE DOSE TO RESTART RESPIRATION AND *B* THE DOSE TO CAUSE OVER-STIMULATION

Thiopentone anaesthesia				Pentobarbital anaesthesia			
Animal No	Picrotoxin dose mg./kg.		Result of observation for 2 hr	Animal No	Picrotoxin dose mg./kg.		Result of observation for 2 hr
	<i>A</i>	<i>B</i>			<i>A</i>	<i>B</i>	
1	1.4	2.5	Lived	11	10.4	14.4	Lived
2	2.0	2.8	Lived	12	9.8	14.1	Lived
3	2.4	10.8	Lived	13	5.2	11.8	Lived
4	2.8	6.8	Lived	14	6.0	11.5	Lived
5	2.5	7.6	Lived	15	5.8	11.9	Lived
6	—	—	Failed to recover	16	1.6	17.4	Lived
7	6.0	13.7	Lived	17	5.5	15.0	Lived
8	6.3	14.1	Lived	18	7.4	17.7	Died
9	5.0	6.8	Lived	19	5.3	12.7	Lived
10	4.8	10.4	Lived	20	5.6	17.8	Died
Mean	3.5	8.3	9/10 lived	Mean	6.28	14.43	8/10 lived
Std. error	0.183	1.40		Std. error	0.92	0.79	

156 or cyclohexyl-ethyl-triazol, also known as "azoman," was described by Behrens, Dinkler, and Woelckhaus (1937). This drug resembles leptazol in many respects, but is much more potent.

The results can also be represented graphically as shown in Figs 1 and 2. These graphs were obtained by plotting the logarithm of the dose required by any one guinea-pig either to restart respiration (*A* curves) or to cause over-stimulation (*B* curves) against the probit value for the percentage of the total number of animals which responded to that dose of the stimulant drug or to smaller doses. The mean value corresponds statistically to the LD50 in a toxicity test and causes recovery in 50 per cent of the animals. The relative doses of the analeptics required can be seen from the graphs, the steepness of the curves gives a measure of the efficiency with which the drugs antagonize the anaesthetics. In Table IV the ratios of the potencies of the three analeptics are given,

taking picrotoxin doses as 1.0. It will be seen that, with thiopentone anaesthesia, both the *A* and the *B* values for triazol 156 bear a constant ratio of 0.47 to the corresponding values for picrotoxin. This is because both drugs cause over-stimulation by the same proportional increase in the doses required to restart the respiration. This is not so with pentobarbital anaesthesia: picrotoxin causes over-stimulation quite readily, but triazol gives a very much smaller ratio for over-stimulation because it does not antagonize this long-acting barbiturate as readily as picrotoxin does.

TABLE IV

A COMPARISON OF THREE ACCEPTED ANALEPTICS. THE VALUES UNDER *A* GIVE THE MEAN DOSES REQUIRED TO RESTART RESPIRATION AND UNDER *B* TO CAUSE OVER-STIMULATION. THE RATIOS ARE THE INVERSE RATIOS OF THE DOSES, TAKING PICTROTOXIN AS 1.0.

Analeptic drug	Thiopentone anaesthesia						Pentobarbital anaesthesia					
	<i>A</i>		<i>B</i>		No alive	Notes	<i>A</i>		<i>B</i>		No alive	Notes
	Dose mg/kg	Ratio	Dose mg/kg	Ratio			Dose mg/kg	Ratio	Dose mg/kg	Ratio		
Picrotoxin 2.0 mg./c.c. 0.6 c.c./min.	3.5	(1.0)	8.3	(1.0)	9/10	<i>a</i>	6.3	(1.0)	14.4	(1.0)	8/10	<i>b</i>
Triazol 156 4.0 mg./c.c. 0.6 c.c./min.	7.5	0.47	17.8	0.47	10/10	<i>c</i>	6.0	1.05	46.4	0.31	4/10	<i>d</i>
Leptazol 100 mg./c.c. 0.6 c.c./min.	218.0	0.016	—	—	9/10	<i>e</i>	234.0	0.027	—	—	4/8	<i>f</i>

(a) Muscular twitching persisted for 5–10 min.

(b) Slight muscular twitching after injection.

(c) Very smooth respiration without twitching. Quick awakening.

(d) Difficult to produce over-stimulation.

(e) No over-stimulation possible. Awakening effect most marked.

(f) No over-stimulation possible.

It will be seen from Table IV and Figs 1 and 2 that the most effective analeptic available to antagonize the barbiturate anaesthetics employed is picrotoxin, but that triazol 156 is not greatly inferior. Picrotoxin is effective against both the short- and long-acting barbiturates used, but leptazol is much less effective against the long-acting drug. Triazol 156 is intermediate between the two and antagonizes pentobarbital far less easily than it does thiopentone anaesthesia. There is much more variability in the results giving the *B* curve for triazol and pentobarbital anaesthesia, and hence the slope of the curve is lower.

Leptazol is the least effective of these stimulant drugs and it did not cause over-stimulation in these experiments. If the injection is continued for a prolonged period, however, secondary depression occurs, when this was observed the injections were stopped one minute after respiration had been re-established.

This secondary depression has also been observed by Das (1939) When leptazol is effective, however, it reduces the anaesthetic duration most markedly. Animals frequently appeared quite conscious within about ten minutes of injecting the drug, although a relapse into deep anaesthesia was common, especially when

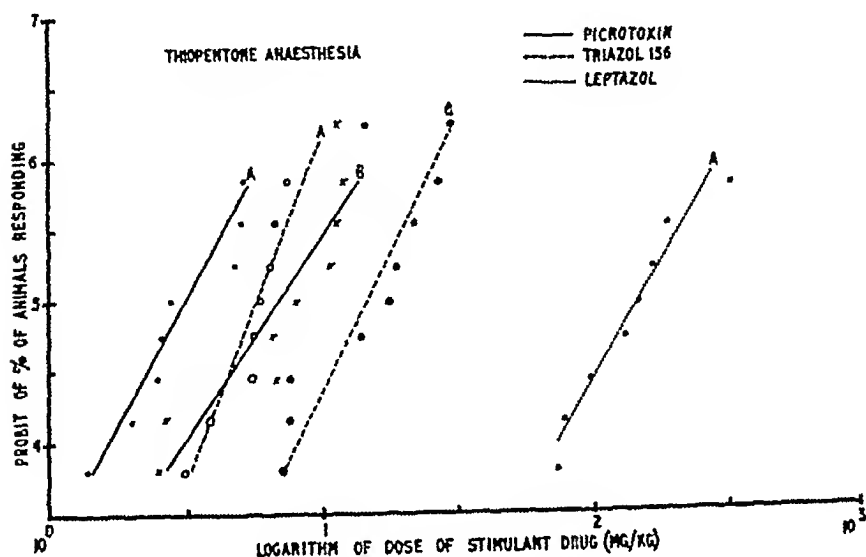


FIG. 1

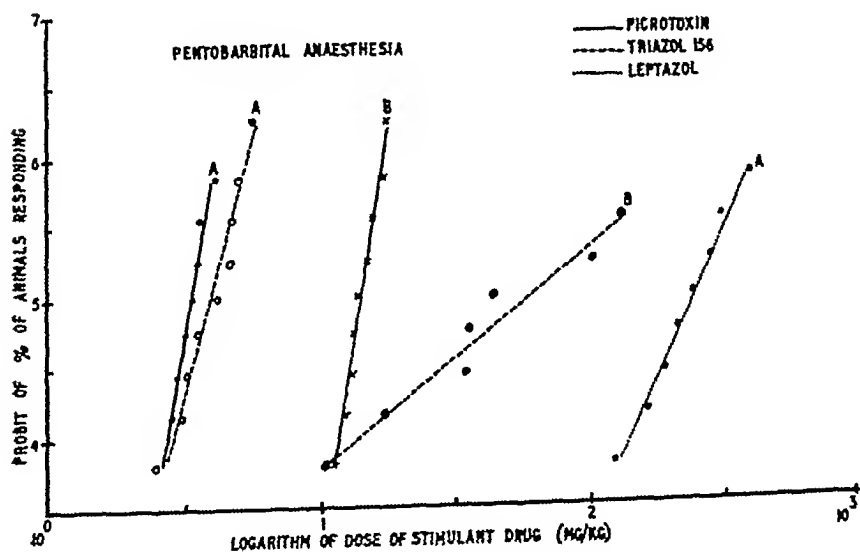


FIG. 2.

pentobarbital was used. This awakening effect was noted by Trevan, and can, in the mouse tests, give a false impression of the efficiency of this drug. Probably the best analeptic of the three tested is triazol 156, since it does not cause the muscular jerking shown by picrotoxin, although larger doses are required.

The method has been used to examine a series of synthetic compounds the results of which will be reported shortly. A compound can be examined in approximately three hours, and the method gives a result under conditions comparable with those of the projected therapeutic application. The method gives no numerical index of the duration of action of these drugs, but transient stimulation while injection is being performed has frequently been observed with new compounds. Whenever this test has indicated useful analeptic activity it has been our practice to use the method described by Das in order to assess the duration of action of the drug, given by various routes, but the method described here provides a valuable primary measure of stimulant activity.

SUMMARY

A method of comparing the respiratory stimulant properties of analeptics is described. Guinea-pigs are anaesthetized by a continuous infusion of short- or long-acting barbiturates, when respiration ceases for twenty seconds the analeptic is also infused. The doses of analeptic required both to restart respiration and to cause over-stimulation are measured.

A comparison of picrotoxin, leptazol, and triazol 156 by this method is described.

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THE BIOLOGICAL ESTIMATION OF SUBSTANCES USED IN TREATING CESTODE INFESTATIONS

BY

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The first experimental observations of the action of anthelmthic drugs were made by von Schroeder (1884, 1885) on annelid worms. He was followed by Trendelenburg (1916), who used round worms, but mainly worked with annelids. Sollmann (1919) and Wasicky (1923) used annelid worms and fish. Hall (1921) was the first to carry out experiments on infested animals. He determined the percentage of parasitic worms which were expelled when dogs were given anthelmthics, his main interest, however, was in nematodes. Rebello, Gomes da Costa, and Toscano Rico (1928, also Gomes da Costa, 1931) showed that cestodes and nematodes behave differently when treated with anthelmthics outside the body, as might be expected since they belong to different phyla of the animal kingdom. Gomes da Costa and his colleagues have developed a method for the observation of isolated segments of *Taenia saginata* and *Dipylidium caninum*. Their results have confirmed clinical findings and they have been able to recommend new drugs for trial (Gomes da Costa, 1930, 1932, Gomes da Costa and Hamet, 1935, 1937). They showed that the survival time of a segment of *Dipylidium caninum* in an oil bath bore an inverse relation to the concentration of filix mas, but made no further attempt to obtain a response graded to the dose (Ettisch and Gomes da Costa, 1937).

Culbertson (1940) worked with mice infested with *Hymenolepis fraterna* (called in this paper *Hymenolepis nana*) and found that doses of atabrine (mepacrine) greatly reduced the number of worms in the intestines of the mice. Again, however, no quantitative results were obtained.

The experiments described in this paper were undertaken to find a convenient method of determining anticestode activity as part of an attempt to isolate the active principles of certain crude drugs. The work has been done with one sample of extractum filicis B.P. Mice supplied to the laboratory were found to be naturally infested by two species of tapeworm, *Hymenolepis nana* and the much larger *Hymenolepis diminuta*. These two species cannot easily be distinguished by inspection of their eggs, so that infested mice may harbour either or

both species, but since *H. diminuta* is so much larger than *H. nana*, the results, which were obtained by weighing the worms, depend almost entirely on the infestation with *H. diminuta*. When fresh mice came to the laboratory, infestation was determined by microscopic examination of the faeces for eggs, as a rule about 30 per cent of the mice were infested. The mice in the faeces of which no eggs were detected were returned to the laboratory stock and were re-examined at intervals of about 3 weeks, when a further 10 per cent were found to be infested.

DETAILS OF PROCEDURE

Examination of mice for infestation.—The mice were fed at least an hour before examination. A pellet of faeces was then taken from each mouse and a faecal smear was made on a microscope slide. Using low power focused on the bottom of the smear, it was fairly easy to spot the typical egg (see Fig 1) containing a hexacanth embryo. When in doubt the high power was used.

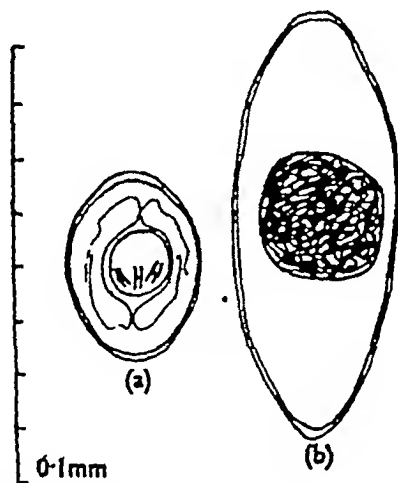


FIG 1—Helminth eggs found in mouse faeces. (a) *Hymenolepis* egg containing a hexacanth embryo. (b) Nematode egg. This is the only common egg likely to be mistaken for *Hymenolepis*.

When ext. filicis is used clinically, the patient is starved overnight. The next morning he is given 3–6 ml. of the extract, followed by a saline purge after two hours. This procedure was adopted with the infested mice, and the proportion of worms excreted was found. Preliminary experiments were carried out to find suitable doses of ext. filicis and of purgative.

Oral toxicity of ext. filicis.—The mouse dose, corresponding to a human dose of 6 ml., is 0.002 ml. An aqueous emulsion was made by shaking ext. filicis with sodium glycocholate (1 per cent) solution. This emulsion was not very stable, but was used for the first experiments. Later, when quantitative results were required, a standard emulsion of ext. filicis in ether and sodium glycocholate solution was prepared as described below.

Groups of mice were given by mouth doses of ext. filicis emulsion (1 and 10 per cent by volume) After 24 hours the dead mice were counted The results are given in Table I.

TABLE I
ORAL TOXICITY OF EXTRACTUM FILICIS

Dose	Volume of emulsion administered	Result
50 mg.	0.5 ml 10 per cent (v/v)	9 out of 15 mice died
20 mg.	0.2 ml 10 " "	0 " 3 " "
10 mg	1.0 ml 1 " "	0 " 3 " "

Since the lethal dose was about 50 mg, one fifth of this dose (10 mg. ext. filicis) was used as the highest therapeutic dose

Choice of purgative—It is usually taught that a saline purgative should be given after ext. filicis, since when castor oil is used the extract dissolves in the oil and is absorbed. The first experiments were therefore made with sodium sulphate as a purgative, this was found to produce diarrhoea when 0.1 g. (anhydrous) was given orally in 1 ml, half this dose was not effective. Since the purgative effect was delayed in some experiments and might have been due to hypertonicity, 1 ml of a solution of 6.5 g $MgSO_4 \cdot 7H_2O$ in 100 ml water, which is isotonic, was used instead.

Examination of material—Examination of faeces or gut contents was carried out in water on a black developing dish. The white fragments of worms were picked out with forceps and rinsed free of debris. At first all fragments were examined microscopically and the number of scolices of each species (*H. diminuta* and *H. nana*) was counted. It was then decided to weigh the worm fragments in a small Gooch crucible after they had been roughly dried with cotton wool and filter paper. An attempt to determine the dry weight after heating to 100°C for 1 hour was unsuccessful because this weight was never more than a few milligrammes and consequently was too small to be an accurate measure of worm content. The wet weight was of the order of 100 mg per mouse and was adopted as a convenient measure of the amount of worms. The results of an early experiment are given in Table II.

TABLE II

Material	Scolices			Weight of worm fragments	
	<i>H. nana</i>	<i>H. diminuta</i>	Total	Wet, mg.	Dry, mg.
Faeces					
10 Treated mice	5	18	23	711	44
10 Control mice	1	0	1	33	<1
Gut contents					
10 Treated mice	2	4	6	167	3
10 Control mice	122	13	135	787	38

Percentage of worms (wet weight) discharged by

(1) Treated mice 81

(2) Control mice 4

In the early experiments the control mice often died, and the conclusion was drawn that the period without food was too long. Glucose injections were first tried as a means of providing calories without giving solid food, but these made no difference. Lumps of sugar were then put in the boxes, and this device was successful, the experiments were carried through without deaths in the control or treated mice provided that mice of not less than 18 g weight were used.

The next difficulty was that mice receiving a high dose of ext. filicis often discharged a smaller percentage of tapeworms than mice receiving a lower dose. It was assumed that the worms were affected by the higher dose, but the mice were weakened and so did not excrete them in the five hours of the experiment. The solution of this problem was suggested by the fact that worms from the intestines of mice in control groups moved vigorously in water and could easily be separated from those which did not move and which were assumed to be killed or paralysed by the drug. To give the worms every chance of movement, the intestines were opened in warm saline. The "dead" worms and live worms from each group were picked out as described before and weighed separately. This division could always be made without hesitation when the worms were touched with forceps. As well as the "dead" worms in the small intestine, worms were sometimes found in the large intestine, including the caecum, these were obviously about to be excreted, because *Hymenolepis* does not inhabit this part of the gut, and they were included in the total of affected worms.

This revised technique was used in eleven experiments. In each experiment groups of five infested mice (each mouse weighing over 18 g), which had been fed on lumps of sugar from 6 p.m. the previous day, were given doses of freshly prepared filicis emulsion, followed by magnesium sulphate as a purgative after two hours. Three hours later the faeces of each group were examined as already described. Each mouse was then killed and its intestine was removed. The small intestine was put into warm (38°C) saline in a black dish and slit open longitudinally with scissors. The mucosa was scraped with a microscope slide and the intestinal contents were examined as described above. The large intestine was treated in the same way. The three categories of worms from the intestines of each group were weighed separately. The weight of worms affected by the drug was expressed as a percentage of the total weight of worms in each group. The method is illustrated by the following experiment.

Exp. 17 Preparation of ext. filicis emulsion—After being stirred the ext. filicis (230 mg., or four drops) was weighed in a 50-ml conical flask and dissolved in 1 ml of ether, distilled water, containing about 1 per cent sodium glycocholate, was added so that 1 ml. of the resulting emulsion contained exactly 10 mg ext. filicis. The emulsion was shaken before use and diluted when necessary with a solution of glycocholate and ether in the same proportions as above so that a volume of 1 ml was administered to each mouse.

The ext. filicis emulsion and the purgative were administered with a blunted hypodermic needle, used as a stomach tube and a 1-ml syringe.

Timetable of experiment

9 40 a.m.	Group 1	5 mice given by mouth	10 mg. ext. filicis
	Group 2.	" "	5 mg. ext. filicis
	Group 3	" "	2.5 mg. ext. filicis
	Group 4	" "	1.25 mg. ext. filicis

Each group was put into a wooden box with a metal floor and supplied with 4 lumps of sugar and water

11 40 a.m. Each mouse was given by mouth 1 ml. warm 6.5 per cent (w/v) magnesium sulphate solution. The groups were replaced in their boxes:

2 40 p.m. Examination of faeces scraped from the floor of each box
Examination of intestines

The results are given in Table III

TABLE III
Figures are weights of worms (mg.)

Dose of ext. filicis	Group 1	Group 2	Group 3	Group 4
	10 mg.	5 mg.	2.5 mg.	1.25 mg.
Worms in faeces (a)	53	235	286	121
Live worms in small intestine (b)	0	75	290	771
"Dead" worms in small intestine (c)	0	41	0	0
Worms in large intestine (d)	65	130	115	0
Total worms (a + b + c + d)	118	481	691	892
Total worms affected by drug (a + c + d)	118	406	401	121
Percentage wet weight of worms affected by drug	100	85	58	13

The results of the eleven experiments are given in Table IV. A total of 220 mice were used, 55 for each dose of ext. filicis

TABLE IV
PERCENTAGE WET WEIGHT OF TAPEWORMS AFFECTED BY DOSES OF EXT. FILICIS
GIVEN TO GROUPS OF 5 MICE

Dose of ext. filicis	Group 1	Group 2	Group 3	Group 4
	10 mg.	5 mg.	2.5 mg.	1.25 mg.
Experiment 14	100	55	48	0
15	91	18	9	0
16	100	88	38	6
17	100	85	58	13
18	100	59	90	0
19	67	85	0	0
20	100	33	67	44
21	96	100	8	0
22	100	45	58	52
23	86	92	40	28
24	85	100	92	14
Mean	92	69	46	14

It will be noticed that in any single experiment (e.g., expts 18, 19) the response is not necessarily graded to the dose, but if the results of any three consecutive experiments are taken together the mean percentage wet weights of tapeworms affected are graded to the doses. This shows that at least fifteen mice must be used for every dose of anticestode drug. The results are shown graphically in Figs 2 and 3. In Fig. 2 the mean percentage effect was plotted against

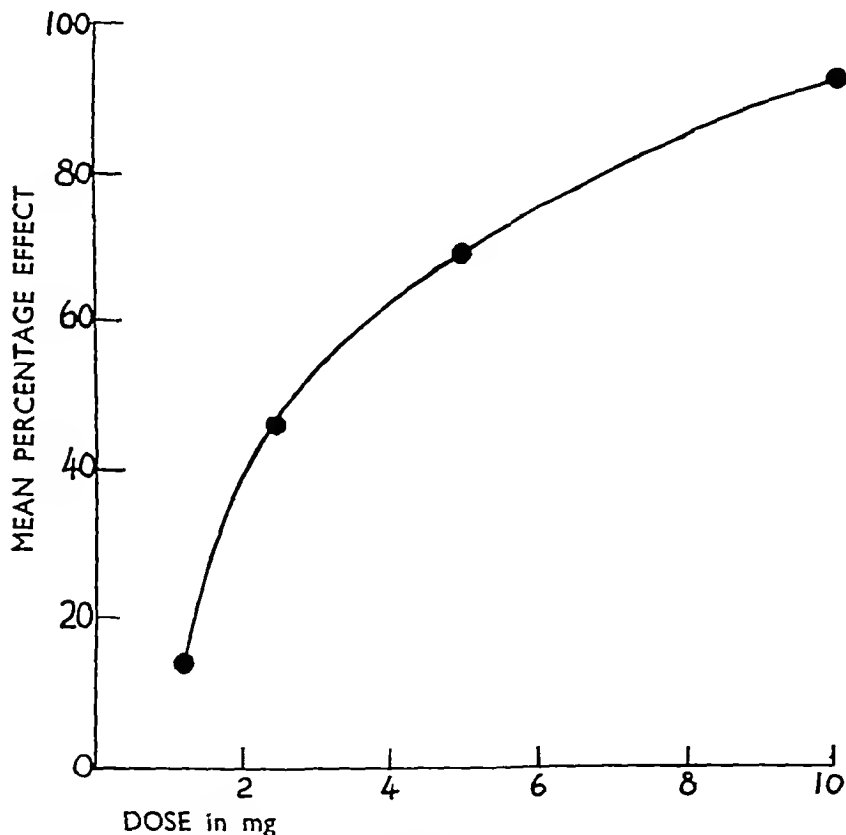


FIG. 2.—Results of 11 experiments using a total of 55 mice for each dose of ext. filicis. Ordinates: Mean percentage wet weight of tapeworms affected. Abscissae: Dose (mg.) given to each mouse.

each dose and a curve was drawn relating dose to effect. In Fig. 3 the expected probits of the mean percentage effect, calculated as described by Bliss (1938), were plotted against the logarithms of the doses. The slope of the line, $b=2.28$. The probability of the line, P , is approximately 0.02.

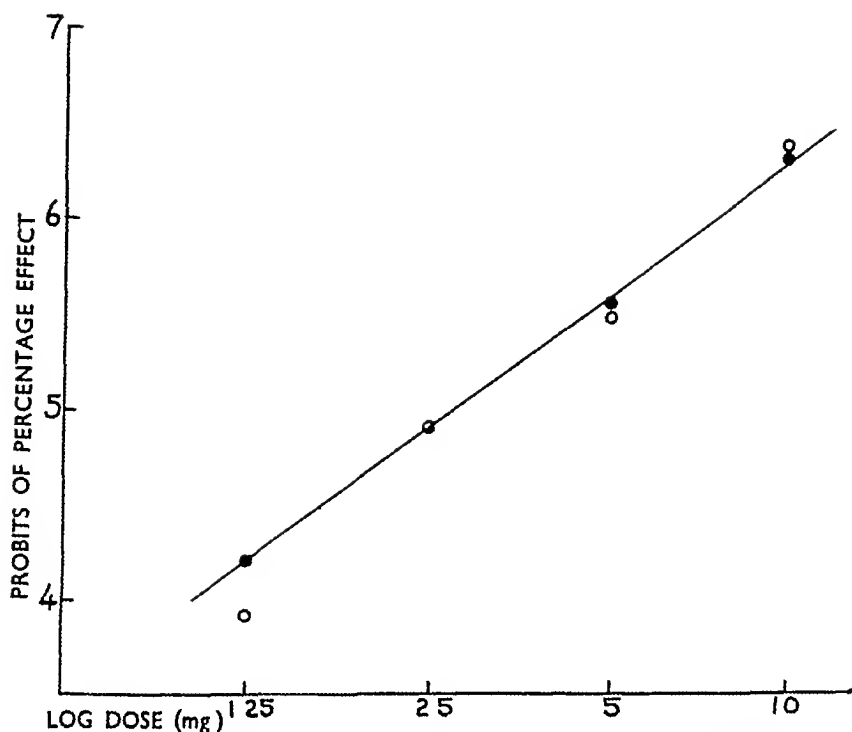


FIG 3—Same results as in Fig 2. Ordinates: Probits of mean percentage wet weight of tapeworms affected. Abscissae: Log dose (mg) given to each mouse. White circles: Empirical probits. Black circles: Expected probits.

SUMMARY

A method for the standardization of anticestode drugs is described. Mice infested with *Hymenolepis diminuta* were used as experimental animals. The mice were fed on lumps of sugar only for 15 hours. Then each mouse was given a dose of ext. filicis in 5 per cent ether emulsion using a stomach tube. Two hours later each mouse was given a dose of magnesium sulphate solution as a purgative. After another three hours the faeces were examined for *Hymenolepis* fragments, which were collected and weighed. The mice were killed and worms from each group were divided into three categories, which were weighed separately.

- 1 Worms from the large intestine (not normally inhabited by *Hymenolepis*)
- 2 "Dead" worms from the small intestine
- 3 Live worms from the small intestine

The weight of the affected worms (categories 1 and 2 and worms in the faeces) was expressed as a percentage of the total weight of worms. Using four different

doses of ext. filicis in a total of 220 mice (55 per dose), it was found that the probits of the percentage wet weight of worms affected increased in linear relation to the logarithm of the dose

This work was carried out as a result of an inquiry from the Colonial Products Research Council whether substances could be tested for activity against tapeworms. The work has been done at the suggestion and under the supervision of Prof J H Burn. My thanks are due to Prof R T Leiper, who very kindly arranged for me to be shown the details of *Hymenolepis* infestation in mice.

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THE AFFINITY OF ATROPINE-LIKE ESTERS FOR ESTERASES

BY

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(Received December 27 1946)

In a recent paper from this department observations on a number of esters with atropine-like actions were reported (Ing, Dawes, and Wajda, 1945). The substances studied included the choline esters of benzoic, tropic, and atrolactic acid, as well as esters of benzoic acid in which choline was replaced by other basic alcohols. In the experiments to be described we have investigated the behaviour of these substances towards esterases, and in particular their affinity for the cholinesterases, by testing whether or not they inhibited the hydrolysis of esters known to be substrates of these enzymes. In addition, we have examined two other esters of interest to the pharmacologist (a) cocaine, and (b) the diethylaminoethyl ester of cyclohexyl-phenylacetic acid (trasentin 6H).

Mammalian tissues contain a number of different enzymes which will hydrolyse acetylcholine and other choline esters (Alles and Hawes, 1940; Richter and Croft, 1942; Mendel and Rudney, 1943; Zeller and Bissegger, 1943; Nachmansohn and Rothenberg, 1945). Mendel and Rudney have shown that two different types of cholinesterase can be characterized by using acetylcholine and benzoylcholine as substrates, they distinguish the "true" cholinesterase from the "pseudo"-cholinesterase. The former will hydrolyse acetylcholine only, the latter both acetylcholine and benzoylcholine. We have found Mendel's nomenclature useful in describing our results and it has therefore been employed in this paper. In addition to these two types of enzymes, preparations have been described which will hydrolyse benzoylcholine, but not acetylcholine. The guinea-pig liver (Sawyer, 1945) and the ox kidney (Gunter, 1946) each contain an enzyme of this kind.

Rabbit serum contains an enzyme, tropinesterase, which hydrolyses atropine, and it was naturally of interest to study the behaviour of the synthetic atropine substitutes towards this enzyme. Tropinesterase does not occur in all rabbits, its presence is genetically determined (Sawin and Glick, 1943).

A few observations were made on an enzyme which hydrolyses tropacocaine in horse serum. The occurrence of this enzyme, first reported by Glick and Glaubach (1941), was confirmed.

The tissues and substrates used are listed in Table I and the substances tested in Table II.

TABLE I
LIST OF TISSUES AND SUBSTRATES USED

Species	Tissue	Amount of tissue used	Substrate	Concentration
Dog	Caudate nucleus	5-10 mg.	Acetylcholine bromide	$6 \times 10^{-3}M$
Horse	Serum	0.2 ml.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Guinea-pig	Liver	12.5-25 mg.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Ox	Kidney	1.0-1.3 g.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Rabbit*	Serum*	0.15 ml	Atropine sulphate	1 g./100 ml
Horse	Serum	0.3 ml	Tropacocaine hydrochloride	$10^{-3}M$

* tropinesterase-positive.

TABLE II

	Substance	Structure
Quaternary bases	Lachesme (E3) or benzilyloxyethyl dimethyl-ethylammonium chloride	$\text{Ph}_2\text{C}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NMe}_2\text{Et}\}\text{Cl}$
	Benzilylcholine chloride (C1)	$\text{Ph}_2\text{C}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NMe}_3}\text{Cl}$
	Tropylcholine chloride	$\text{Ph}.\text{CH}(\text{CH}_2\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NMe}_3}\text{Cl}$
	Atrolactylcholine chloride	$\text{Ph}\text{CMe}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NMe}_3}\text{Cl}$
Tertiary bases	Dimethylaminoethyl benzilate hydrochloride (C4)	$\text{Ph}_2\text{C}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NMe}_2\text{H}\}\text{Cl}$
	Diethylaminoethyl benzilate hydrochloride (E1)	$\text{Ph}_2\text{C}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NEt}_2\text{H}\}\text{Cl}$
	Trasentun 6H or diethylaminoethyl phenylcyclohexylacetate hydrochloride	$\text{Ph} \begin{array}{c} \diagup \\ \text{C}(\text{OH})\text{CO}_2\text{CH}_2\text{CH}_2\text{NEt}_2\text{H} \end{array} \begin{array}{c} \diagdown \\ \text{C}_6\text{H}_{11} \end{array} \}\text{Cl}$
	Cocaine hydrochloride	$\begin{array}{c} \text{MeOCO} \\ \diagdown \\ \text{CH}-\text{CH}-\text{CH}_2 \\ \diagup \quad \quad \quad \diagdown \\ \text{Ph.CO}_2\text{CH} \quad \quad \quad \text{HNMe} \quad \quad \quad \text{CH}_2 \\ \diagdown \quad \quad \quad \diagup \\ \text{CH}_2-\text{CH}-\text{CH}_2 \end{array} \}\text{Cl}$
	Tropacocaine hydrochloride	$\begin{array}{c} \text{CH}_2-\text{CH}-\text{CH}_2 \\ \diagup \quad \quad \quad \diagdown \\ \text{Ph.CO}_2\text{CH} \quad \quad \quad \text{HNMe} \quad \quad \quad \text{CH}_2 \\ \diagdown \quad \quad \quad \diagup \\ \text{CH}_2-\text{CH}-\text{CH}_2 \end{array} \}\text{Cl}$

Serial numbers in parentheses are taken from Ing *et al* (1945)

MATERIALS AND METHODS

Enzyme extracts were prepared by grinding the appropriate organs with sand, Krebs's Ringer-bicarbonate was used as the medium. No sand was added in preparing extracts of caudate nucleus, a suspension of the thoroughly ground tissue being used. No attempt was made to purify the enzyme preparations used, and it should be borne in mind that impurities may have influenced the affinities of the substances tested in this and the following paper.

Non-enzymic hydrolysis during the experiments occurred to a very small extent with the quaternary ammonium bases, but benzilic esters with a tertiary basic group showed a larger spontaneous hydrolysis which gave rise to a small blank. Trasentin 6H gave a slight precipitate with the Ringer solution, and its actual concentration is therefore somewhat uncertain.

The hydrolysis was followed manometrically, the experiments were carried out at a temperature of 38° with a gas mixture containing 95 per cent N₂ and 5 per cent CO₂.

RESULTS

(1) *Hydrolysis of acetylcholine by dogs' caudate nucleus* (Table III)—Preparations of the caudate nucleus contain a powerful cholinesterase (Nachmansohn, 1937). This enzyme is a true cholinesterase (Mendel and Rudney, 1943).

Not one of the esters examined was hydrolysed by this preparation. We also tested their affinity for the enzyme by measuring their effect on the rate of hydrolysis of acetylcholine. They had very little action. The strongest effect was found with cocaine, which, in a concentration equimolecular to that of acetylcholine, caused an inhibition of 50 per cent. With trasentin 6H the inhibition was less, and none of the benzilic esters had any inhibitory activity in the concentration used.

TABLE III
EXTRACT OF THE CAUDATE NUCLEUS OF THE DOG
Hydrolysis of acetylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	0
Benzylcholine (C1)	$6 \times 10^{-3}M$	0
Tropylcholine	$6 \times 10^{-3}M$	0
C4	$6 \times 10^{-3}M$	0
E1	$6 \times 10^{-3}M$	0*
Trasentin 6H	$6 \times 10^{-3}M$	15
Cocaine	$6 \times 10^{-3}M$	50

* This experiment was carried out with an extract of rabbit's basal ganglia.

(2) *Hydrolysis of benzoylcholine by horse serum* (Table IV)—Horse serum contains an active pseudo-cholinesterase, and in order to exclude the action of true cholinesterase we used benzoylcholine as substrate. The same sample of serum was used in all these experiments.

Horse serum hydrolysed atrolactylcholine, these observations are described separately in section (6). None of the other esters was hydrolysed, but they all had an affinity for the horse serum esterase: all the substances tested inhibited the enzyme, and with cocaine and trasentin the percentage inhibitions were higher than for true cholinesterase.

TABLE IV
HORSE SERUM
Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	71
Benzilylcholine (C1)	$6 \times 10^{-3}M$	33
Tropylcholine	$6 \times 10^{-3}M$	43
Trasentin 6H	$6 \times 10^{-3}M$	81
Cocaine	$6 \times 10^{-3}M$	85

(3) *Hydrolysis of benzoylcholine in guinea-pig liver* (Table V)—This preparation contains no pseudo-cholinesterase (Blaschko, Chou, and Wajda, 1947), the hydrolysis of benzoylcholine is solely due to the benzoylcholinesterase described by Sawyer (1945). Whether or not benzoylcholine is the normal substrate of the enzyme in the living animal is unknown.

All the esters examined acted as inhibitors of the enzyme (see Table V) and the percentage inhibitions were higher than for either true or pseudo-cholinesterase. Not one of the esters was hydrolysed by the enzyme, with the possible

TABLE V
GUINEA-PIG'S LIVER EXTRACT
Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	94
" "	$6 \times 10^{-4}M$	91
" "	$6 \times 10^{-5}M$	66
C4	$6 \times 10^{-3}M$	100
E1	$6 \times 10^{-3}M$	100
Trasentin 6H	$1.2 \times 10^{-3}M$	97
" "	$1.2 \times 10^{-4}M$	88
" "	$1.2 \times 10^{-5}M$	80
" "	$1.2 \times 10^{-6}M$	45
Cocaine	$6 \times 10^{-3}M$	100
"	$6 \times 10^{-4}M$	92
"	$6 \times 10^{-5}M$	44

exception of trasentun 6H, where with the lowest concentration used ($1.2 \times 10^{-3}M$) the percentage inhibitions during the first 15 min period were consistently higher than in the second

(4) *Hydrolysis of benzoylcholine in ox kidney* (Table VI)—The benzoylcholinesterase of ox kidney described by Gunter (1946) has not yet been fully analysed. The enzymic activity of the tissue extracts is very much less than that of the extracts of guinea-pig liver, and it was therefore necessary to use much larger amounts of tissue (see Table I)

Our results with this preparation showed a striking difference from those with the guinea-pig liver extract the esters examined had little or no effect on the rate of hydrolysis of benzoylcholine

TABLE VI
OX KIDNEY EXTRACT
Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	12
Benzilylcholine (Cl)	$6 \times 10^{-3}M$	19
C4	$6 \times 10^{-3}M$	0 (approx.)
E1	$6 \times 10^{-3}M$	29 (approx.)
Trasentun 6H	$10^{-3}M$	0
Cocaine	$10^{-3}M$	14

(5) *Hydrolysis of atropine in rabbit serum* (Table VII)—All experiments were carried out with the sera of two animals which were found to contain tropinesterase, they were among a group of about a dozen animals tested for the presence of the enzyme. Not one of the substances included in Table VII showed any enzymic hydrolysis. Cocaine was not included, as it was found to be hydrolysed enzymically, we confirmed Ghick and Glaubach's (1941) observation that this hydrolysis also occurs in the serum of animals without tropinesterase.

The benzilic esters had no inhibitory action on tropinesterase (see Table VII), of all the substances examined only trasentun 6H had a slight inhibitory effect.

TABLE VII
RABBIT'S SERUM
Hydrolysis of atropine sulphate (1 g/100 ml.) by tropinesterase

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-3}M$	0
Benzilylcholine (Cl)	$10^{-3}M$	0
Atrolactylcholine	$6 \times 10^{-3}M$	0
Trasentun 6H	$10^{-3}M$	60

(6) *Enzymic hydrolysis of atrolactylcholine in horse serum*—None of the synthetic esters examined showed any detectable enzymic hydrolysis with the exception of the choline ester of atrolactic acid in horse serum. Only small amounts of the substance were at our disposal, but the following facts were established: the ester showed an appreciable hydrolysis in Ringer solution, but the rate of liberation of carbon dioxide was consistently higher in the presence of horse serum. That this hydrolysis in excess of the blanks was due to an enzyme is supported by the following observations:

(i) the hydrolysis increased with increasing amounts of serum, in one experiment the additional amount of CO_2 liberated in 15 min. by 0.2 ml. of horse serum was 9.5 μl ., in another experiment with 0.4 ml. of serum it was 19 μl (the figures for spontaneous hydrolysis in the blanks were 7.5 and 9.5 μl . respectively),

(ii) the additional hydrolysis was abolished by boiling the serum, and

(iii) it was reduced in the presence of $1.8 \times 10^{-3}M$ eserine.

In addition the following facts were established: there was no enzymic hydrolysis of atrolactylcholine in rabbit serum (0.15 ml. per flask) and in dog's caudate nucleus extract (equivalent of 40 mg. fresh weight of tissue per flask).

These results strongly suggest that atrolactylcholine is a substrate of pseudo-cholinesterase. Mendel *et al* (1943) have shown that the serum of the horse has about 5 times as much pseudo-cholinesterase activity as that of the rabbit. Moreover, the eserine inhibition of the hydrolysis of both benzoyl- and atrolactylcholines were of the same order: in the presence of $1.8 \times 10^{-3}M$ eserine the hydrolysis of $6 \times 10^{-3}M$ benzoylcholine was reduced, the CO_2 output falling from 102 to 38.5 μl in 15 min., and for atrolactylcholine, used in the same concentration, the corresponding figures were 15 and 9 μl CO_2 respectively. It was also shown that atrolactylcholine had an affinity to pseudo-cholinesterase, as the hydrolysis of benzoylcholine was reduced in the presence of the atrolactic ester. The amounts of carbon dioxide liberated by 0.2 ml. of horse serum in 15 min. were

with $6 \times 10^{-3}M$ benzoylcholine—117 μl
 with $6 \times 10^{-3}M$ atrolactylcholine—9.5 μl
 with both esters—58.5 μl .

(7) *Hydrolysis of tropacocaine in horse serum* (Table VIII)—None of the substances examined had any affinity for the enzyme in the concentrations used.

TABLE VIII
 HORSE SERUM
 Hydrolysis of tropacocaine ($10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-3}M$	0
Benzylcholine (C1)	$10^{-3}M$	0
Cocaine	$10^{-3}M$	0
Atropine	$10^{-3}M$	0

in our experiments. The enzyme responsible for the hydrolysis of tropacocaine must therefore be distinct from pseudo-cholinesterase, as this enzyme is inhibited by some of the substances listed in Table VIII, e.g., cocaine, lachesine, and benzylcholine.

DISCUSSION

Our observations provide an example of the usefulness of inhibitors for distinguishing related enzymes. All the enzymes studied were esterases and yet great differences in their affinities to the esters tested were found. The degree of inhibition is determined not by the substrate used, which may or may not be common to two enzymes, but by the specific affinities of the enzyme itself. With acetylcholine as substrate this was first clearly demonstrated in experiments by Zeller (1942), who found that the human serum cholinesterase was much more sensitive to isopropyl-antipyrin than the cholinesterase of the central nervous system.

Our results can be summarized in the following scheme

Substrate	Acetylcholine		Benzoylcholine	
Enzyme	True cholinesterase	Pseudo-cholinesterase	Benzoylcholinesterase of guinea pig liver	Benzoylcholinesterase of ox kidney
Affinity to the esters tested	low	intermediate	high	low

All the substances tested had little affinity to true cholinesterase, more to pseudo-cholinesterase, and the highest affinity to the benzoylcholinesterase of guinea-pig liver. The benzoylcholinesterase of ox kidney was little or not at all inhibited. The activity of the ox kidney was very low and the amount of tissue used had therefore to be very much greater than with any of the other preparations (Table I). The possibility that the small inhibitor action in this case is due to a reaction of the esters with some other constituents in the ox kidney cannot therefore be excluded, but it seems more likely that the benzoylcholinesterase of ox kidney differs from the corresponding enzyme in the liver of the guinea-pig.

We have not found any enzymic hydrolysis of lachesine or any of the other esters of benzoic acid tested as atropine substitutes by Ing *et al* (1945). It is interesting that these substances which must have a great affinity for the tissue receptors on which atropine acts have little or no affinity for the enzyme tropinesterase.

Our experiments on atrolactylcholine increase the number of choline esters known to be hydrolysed in animal tissue, it seems interesting that the chemically closely related esters of tropic and benzoic acids were not hydrolysed

Cocaine, an ester of benzoic acid, has a much higher affinity for pseudo-cholinesterase, which hydrolyses the benzoic ester of choline, than for the true esterase which does not (see also Nachmansohn and Schneemann, 1945). Cocaine also inhibits the benzoylcholinesterase of guinea-pig's liver, but neither the benzoylcholinesterase of ox kidney nor the tropacocainesterase of horse serum was inhibited by cocaine, this shows that affinity for cocaine is not a general property of esterases which hydrolyse esters of benzoic acid

SUMMARY

1 The action of cocaine, benzilylcholine, lachesine, and a number of related atropine-like esters on cholinesterases and on tropinesterase has been studied

2 These substances were found to have little affinity to the "true" cholinesterase of brain tissue, more affinity to the "pseudo"-cholinesterase of horse serum, and a high affinity to the benzoylcholinesterase of guinea-pig's liver. They had little or no effect on the hydrolysis of benzoylcholine by ox kidney extracts

3 They had little or no affinity to the tropinesterase of rabbit's serum

4 Evidence is given which suggests that atrolactylcholine is hydrolysed by pseudo-cholinesterase of horse serum

5 The tropacocainesterase of horse serum was not inhibited by these substances, which shows that it is not identical with pseudo-cholinesterase

We are grateful to Dr H R Ing for the benzoylcholine and the esters prepared by Ford-Moore and Ing (1947), and to Dr R Wien for the tropacocaine used in these experiments

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THE INHIBITION OF ESTERASES BY PALUDRINE

BY

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Many observations on the action of antimalarial drugs on ester-splitting enzymes have been recorded. Rona and Reinicke (1921) have shown that quinine inhibits the esterase which hydrolyses tributyrin in human serum, this enzyme is also inhibited by many other antimalarials, e.g. pamaquin, optochin, and mepacrine (Fulton, 1936). Both quinine and mepacrine act on cholinesterases: quinine has been shown to inhibit the enzyme now generally known as pseudo-cholinesterase (Nachmansohn and Schneemann, 1945), mepacrine inhibits the hydrolysis of acetylcholine by both pseudo- and true cholinesterases (Waelesch and Nachmansohn, 1943).

It therefore seemed interesting to examine the effect of paludrine on esterases. Paludrine differs greatly from the other antimalarials in its chemical constitution and is characterized by a low toxicity. The latter fact made it unlikely that it would strongly inhibit an enzyme so vital as the "true" cholinesterase of the central nervous system and muscles, but it is known that there are other enzymes which will hydrolyse choline esters. We have therefore examined the action of paludrine on all the known cholinesterases and on a number of related enzymes.

MATERIAL AND METHODS

Most of these have been described in the preceding paper (Blaschko, Chou, and Wajda, 1947). In addition we have examined the hydrolysis of acetylcholine by plasma and red-cell haemolysate. The hydrolysis of tributyrin was also studied in human serum and in cat's liver extracts. The cat's liver used for these experiments was washed free from blood *in situ* by perfusion with Locke's fluid.

EXPERIMENTS

1 Cholinesterases (Table I)

Two preparations, representative of true cholinesterase, were examined: an extract from the dog's caudate nucleus and a haemolysate of human red cells. Both were inhibited slightly by paludrine. The rate of hydrolysis of acetylcholine ($6 \times 10^{-4}M$) by the brain extracts was reduced by about 22 per cent in the presence of $10^{-3}M$ paludrine. For the human haemolysate the inhibition was 49 per cent with $10^{-3}M$ paludrine, with $10^{-4}M$ paludrine there was no inhibition.

In the plasma (or serum) of many mammals the enzyme acting on acetylcholine is pseudo-cholinesterase. This enzyme which hydrolyses benzoylcholine as well as acetylcholine was more strongly inhibited by paludrine. In one experiment with human plasma and acetylcholine as substrate the percentage inhibitions were 92 per cent with $10^{-3}M$, and 60 per cent with $10^{-4}M$ paludrine. Similar inhibitions were observed with cat's plasma and horse serum as sources of the enzyme.

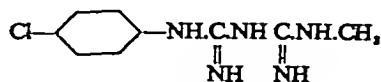
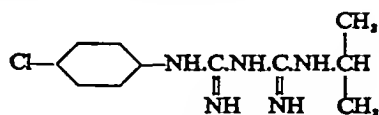
TABLE I
ACTION OF PALUDRINE ON CHOLINESTERASES
Substrate concentration $6 \times 10^{-4}M$

Species and tissue	Amount of tissue	Substrate used	Percentage inhibition by paludrine		
			$10^{-3}M$	$10^{-4}M$	$10^{-5}M$
Dog's caudate nucleus	4 mg.	acetylcholine	22	—	—
Human red-cell haemolysate	0.1 ml.	"	49	0	—
Human plasma	0.05 ml	"	92	60	—
Horse serum	0.3 ml	benzoylcholine	67	35	—
Cat plasma	0.005 ml	acetylcholine	73	—	—
Guinea-pig liver	12.5 mg.	benzoylcholine	94	76	26
Ox kidney	1,000 mg.	"	0	—	—

Benzoylcholinesterase hydrolyses benzoylcholine, but not acetylcholine, the enzyme has been found in the liver of the guinea-pig (Sawyer, 1945) and in ox kidney (Gunter, 1946). It is known that the latter organ does not contain pseudo-cholinesterase, and our experiments show that the same is true for the guinea-pig's liver. This was established by comparing the rates of hydrolysis of benzoylcholine with and without eserine, which is a strong inhibitor of pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943). We find that the rate of hydrolysis of benzoylcholine is the same in the presence or absence of 2×10^{-4} eserine, a concentration which completely inhibits pseudo-cholinesterase.

The affinities of paludrine for the two preparations of benzoylcholinesterase are very different. Whereas the ox kidney preparation was not inhibited by $10^{-3}M$ paludrine, this concentration rendered the enzyme from guinea-pig's liver almost completely inactive.

It seemed interesting to compare the effect of paludrine on benzoylcholinesterase with that of a related compound which has no antimalarial action, at least on *P. Gallinaceum* in chicks (Curd and Rose, 1946). Such a compound is the N_1 -methyl homologue of paludrine, the substance M5093. In this substance the isopropyl group of paludrine is replaced by a methyl group.



M5093

The anti-benzoylcholinesterase activity of M5093 is very much less than that of paludrine. In one experiment we compared the inhibition with paludrine and M5093, both in $10^{-3}M$ concentrations, on the benzoylcholinesterase of guinea-pig's liver. The inhibitions were

with paludrine 91 per cent, with M5093 42 per cent.

In another experiment the inhibitions were

with $4 \times 10^{-3}M$ paludrine 49 per cent, with $10^{-3}M$ M5093 41 per cent.

The latter experiment shows that the N methyl homologue was less active as an inhibitor when present in a concentration 25 times that of paludrine

M5093 had no inhibitory action on the true cholinesterase from dog's brain in a molar concentration of 10^{-3}

2 Tributyrinesterases (Table II)

Rona and his collaborators have shown that the effect of quinine on the enzymic hydrolysis of tributyrin differs for preparations obtained from different organs the tributyrinesterase of serum is strongly inhibited by quinine (Rona and Reinicke, 1921) but the esterase from liver is resistant to quinine (Rona and Pavlovic, 1922)

Both paludrine and mepacrine behave similarly to quinine In the presence of $10^{-3}M$ paludrine the tributyrinesterase of human serum was almost completely inhibited and with $10^{-4}M$ paludrine the inhibition was 82 per cent. With $10^{-3}M$ mepacrine the inhibition was 92 per cent. On the other hand, with the preparation from cat's liver, the rate of hydrolysis of tributyrin was not affected in the presence of $10^{-3}M$ paludrine and of $10^{-3}M$ mepacrine

It is interesting that the three antimalarial substances, so very different in their chemical constitution, should resemble each other so closely in their affinities for this enzyme, but it must be pointed out that the compound M5093, which is without antimalarial activity, behaves in a similar fashion In a concentration of $10^{-3}M$ it was without inhibitory action on the enzyme from cat's liver, but with human serum the inhibition was 72 per cent.

TABLE II
INHIBITION OF TRIBUTYRINESTERASES
Substrate concentration 0.015 M

Species	Tissue	Amount of tissue	Inhibitor	Percentage inhibition at different concentrations		
				$10^{-3}M$	$10^{-4}M$	$10^{-5}M$
Man	Serum	0.2 ml	paludrine	94	81	24
Cat	Liver	0.667 mg	"	2	—	—
Rabbit	Pancreas	20 mg	"	0	—	—
Rabbit	Kidney	200 mg	"	0	—	—
Man	Serum	0.2 ml	mepacrine	92	87	60
Cat	Liver	0.667 mg	"	0	0	0
Man	Serum	0.2 ml	M5093	73	36	8
Cat	Liver	0.667 mg	"	0	—	—

The hydrolysis of tributyrin in rabbit's kidney and pancreas was not inhibited by $10^{-3}M$ paludrine

3 Other esterases (Table III)

Paludrine had no effect on the hydrolysis of methyl butyrate by rabbit's pancreas It had a slight inhibitory action on the tropinesterase of rabbit's serum with $10^{-3}M$ paludrine the inhibition was 48 per cent We have also examined the tropacocainesterase of horse serum described by Glick and Glaubach (1941), this enzyme was not inhibited by paludrine

TABLE III
ACTION OF PALUDRINE ON OTHER ESTERASES
Paludrine concentration $10^{-3}M$

Species and tissue	Amount of tissue used	Substrate used	Percentage inhibition
Rabbit pancreas	20 mg.	0.015 <i>M</i> methylbutyrate	0
Rabbit serum*	0.15 ml	1 g/100 ml atropine sulphate	48
Horse serum	0.3 ml	0.01 <i>M</i> tropacocaine hydrochloride	0

* The serum was from a rabbit which contained tropinesterase.

DISCUSSION

The mechanism of antimalarial activity is a matter of speculation, but it seems likely that the therapeutic action is due to the drugs interfering with metabolic reactions of vital importance to the parasites, such interference is most easily thought of as an inhibition of enzymic reactions. Plasmodial enzymes have not yet been available for study, but it has long been known that some antimalarials will inhibit certain mammalian enzymes. Paludrine shares this property with such well-known antimalarials as quinine and mepacrine, but also displays some differences, it has, for example, a very low affinity for one of the physiologically most important enzymes, namely the true cholinesterase. Its most powerful inhibitory action in our experiments was on the benzoylcholinesterase of guinea-pig's liver, an enzyme so far characterized by a substrate which has not yet been shown to occur in animal tissues.

There is no general parallelism between antimalarial and anti-esterase properties, but the fact that so many antimalarials are at the same time inhibitors of esterases makes the suggestion that these substances act by inhibiting an esterase of importance in the life cycle of the malaria parasite worthy of investigation.

The pattern of affinities of paludrine for the cholinesterases is similar to that of the substances examined in the preceding paper. Like cocaine, trasentin 6H, and lachesine, paludrine has little affinity for true cholinesterase, pseudo-cholinesterase is more strongly inhibited and there is a strong inhibition of the benzoylcholinesterase of guinea-pig's liver, whereas the benzoylcholinesterase of the ox kidney is not inhibited. So far as true and pseudo-cholinesterase are concerned, it has been shown that many substances have a greater affinity for the latter enzyme, e.g., diisopropyl fluorophosphonate (Hawkins and Mendel, 1947), quinine (Nachmansohn and Schneemann, 1945), and the aromatic ammo alcohols of the type $Ar-CHOH-CH_2-NR_2$, recently studied by Wright (1946). It is

interesting that the enzyme specifically connected with the metabolism of acetylcholine in nerve and muscle should be much less readily inhibited

The authors are grateful to Dr F L. Rose for the samples of paludrine and its N₂-methyl homologue used in this investigation.

Two of us (H B and I W) are indebted to the Medical Research Council for personal grants held while this investigation was carried out.

SUMMARY

- 1 Paludrine has little affinity for true cholinesterase
- 2 It inhibits pseudo-cholinesterase more strongly
- 3 It is an inhibitor of the benzoylcholinesterase of guinea-pig's liver, but not of the benzoylcholinesterase of ox kidney
- 4 Paludrine, like quinine and mepacrine, has little affinity for the tributyrinesterase of cat's liver, but does inhibit the tributyrinesterase of human serum
- 5 The N₂-methyl homologue of paludrine has a much lower affinity for the benzoylcholinesterase of guinea-pig's liver than paludrine itself
- 6 The guinea-pig's liver does not contain pseudo-cholinesterase

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OXIDATION OF ADRENALINE IN ALKALINE SOLUTION

BY

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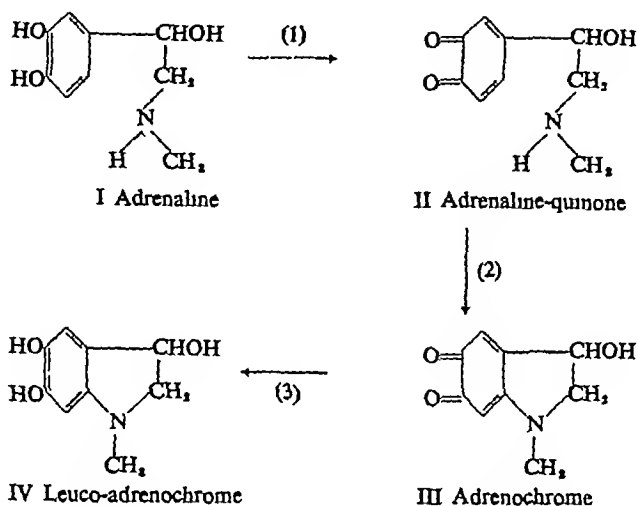
When adrenaline in simple alkaline solutions is exposed to oxygen, the loss of physiological activity is accompanied by various other known phenomena

1 The solution becomes pink and then red owing to the formation of adrenochrome, which has been isolated in crystalline form and has formula III (Green and Richter, 1937, Richter and Blaschko, 1937) Eventually, this indole quinone is destroyed and the red colour disappears

2 A green fluorescence develops and then disappears (for references see Jørgensen, 1945) Attention was drawn to the advantages of this fluorescence for the detection of adrenaline by Gaddum and Schild (1934), who found that it provided a sensitive physical test, by which a concentration of 10^{-8} adrenaline could be easily detected They also found that the reaction was specific at this concentration many substances chemically related to adrenaline giving no fluorescence, whilst others gave fainter green fluorescences in more concentrated solutions

3 The solution acquires an increased power of reducing arsenomolybdic acid (Shaw, 1938) This fact has also been made the basis of a specific and sensitive test for adrenaline If the reducing power of a tissue extract be increased many times by treatment with alkali, the observation can be taken as evidence that the extract contains adrenaline or some other phenol with the same side chain Shaw stated that, if the treatment with alkali was prolonged, the colour decreased and the more active reducing agent was itself destroyed.

It has been suggested (Utevsky, 1944) that adrenaline undergoes the following chemical changes in these conditions



Substance IV eventually undergoes further changes of unknown nature. Substances II and IV have not been isolated, but the theory is supported by a certain amount of indirect evidence.

The object of the experiments described here was to determine the relations which exist between the formation of adrenochrome, the appearance and disappearance of fluorescence, the changes in reducing power and the disappearance of biological activity. In addition, experiments were carried out in order to obtain evidence about the substances responsible for these different effects and to devise an improved method, based on the fluorescence reaction, of detecting adrenaline in low concentrations.

The fluorescence reaction and chemical properties of the oxidation products of adrenaline

Gaddum and Schild showed that oxidation is the cause of the fluorescence of alkali-treated adrenaline, since none was produced if all oxygen was removed from the solution. Utevsky assumed that oxidation is the cause of the weakening as well, since intense oxidation destroys the fluorescent product. By preparing solutions containing large proportions of each of the reaction products, he was able to study some of their properties. He found that the duration of fluorescence when alkali was added to the orthoquinone (II) or to adrenochrome was shorter than when it was added to adrenaline. Formaldehyde inhibited oxidation of adrenaline to adrenaline-quinone before the indole ring had been

formed. On the other hand, he found that strong fluorescence was produced if adrenochrome and formaldehyde together were treated with alkali, suggesting that the fluorescent material was related to adrenochrome. The conclusion reached by Utevsky was that the material giving the fluorescence was leuco-adrenochrome (IV).

In a limited study, the above reactions have been repeated and confirmed, and so far we have found no evidence to disprove Utevsky's conclusions. The fluorescent material is yellow in solution, gives a positive ferric chloride reaction for catechols, a positive *p*-dimethylaminobenzaldehyde test for indoles, and is adsorbed at pH 8.5 by aluminium hydroxide. Efforts to isolate the fluorescent material in the dry state have so far failed, and therefore some of these reactions may be due to impurities. The life of the fluorescent material can be lengthened by the addition of ascorbic acid to a solution with maximal intensity, by adjusting the pH of the solution to 6 after maximal production, or by adsorption on alumina at pH 8.5.

Quantitative estimation of adrenaline

Much work has been done in adapting the fluorescence reaction for quantitative work. The findings described here were obtained before Jorgensen's work was published, but the conclusions are in good agreement with his.

Gaddum and Schild recommended the use of 5*N* NaOH for obtaining the fluorescence, but the interval of maximal intensity was then very short. The reading was taken after 20 seconds contact, and after about one minute no fluorescence remained. The use of more

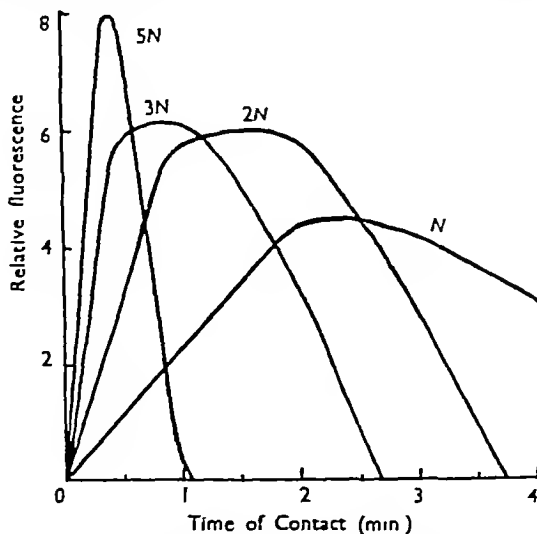


FIG 1—The effect of different concentrations (*N* to 5*N*) of NaOH on the intensity and duration of fluorescence of adrenaline solutions. Ordinates relative fluorescence as measured on the fluorimeter. Abscissae time of contact between adrenaline (10^{-7}) and alkali (1/10 vol.)

dilute alkali was investigated, therefore, in order to prolong this maximum and enable comparisons to be carried out more accurately. The results of this work, shown in Fig. 1, indicated that a satisfactory strength was 2N NaOH, with which maximal fluorescence was obtained in 1 min and lasted for 1 min, only a slight loss of sensitivity resulted. Comparisons were made by two methods in the first (hereinafter referred to as West's method), the adrenaline fluorescence was compared in Rimington's (1943) fluorimeter against standard eosin solutions, whilst the second was a modification of Jørgensen's method, employing one concentrated standard eosin solution. In the fluorimeter, blank readings for water and alkali only were high, and relative fluorescence readings were obtained by subtracting the scale readings from the blank ones. A linear relationship existed between these readings and the concentration of adrenaline in the solution, for each standard eosin solution used (Fig. 2). Fluorescence was produced by treating 3 ml of the adrenaline solution with 0.3 ml of 2N NaOH. The two standard eosin solutions had fluorescent intensities corre-

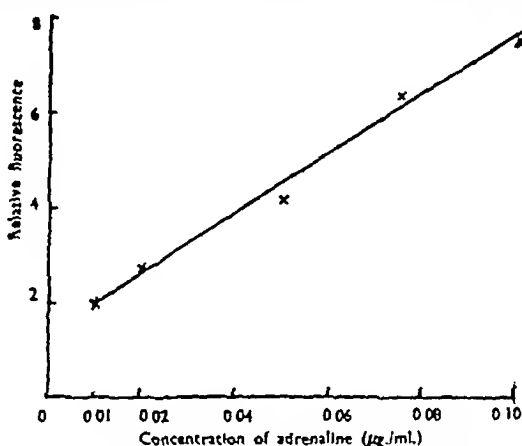


FIG. 2.—Calibration curve for fluorescence of alkali-treated adrenaline, using standard eosin solution "A."

sponding to alkali-treated 10^{-7} and 10^{-8} adrenaline respectively. Monax glass test-tubes have been used throughout this work, as they do not fluoresce in ultra-violet light.

In the second method, the fluorescence was compared with a similar volume of distilled water to which eosin solution (0.1 mg/100 ml.) was added from a burette. Another standard curve was obtained, the volume of eosin solution added being linearly related to the adrenaline content of the solution (Fig. 3). The method was improved by the use of

TABLE I

ESTIMATIONS OF "UNKNOWN" SOLUTIONS OF ADRENALINE BY THE TWO FLUORIMETRIC METHODS. Readings taken after contact with alkali for 1 min. Each experimental figure represents the average of six estimations.

Solution taken	Adrenaline in $\mu\text{g./ml}$									
	1.000	0.800	0.500	0.300	0.200	0.100	0.070	0.050	0.030	0.010
Found, West's method	1.010	0.810	0.476	0.303	0.220	0.100	0.074	0.048	0.030	0.014
" Jørgensen's method	1.020	0.830	0.542	0.300	0.220	0.098	0.075	0.055	0.033	0.012

less alkali (0.2 ml of 2*N* NaOH) and less water (2.0 ml of water), and by carrying out comparisons in the fluorimeter. Solutions of adrenaline prepared by an independent worker were estimated by both methods and good agreement found. The results are recorded in Table I, each figure representing the average of six determinations. The standard error of the test, calculated by the method of Gaddum (1938) using the true values of the 'unknown' solutions, was 2.11 per cent with West's method and 2.15 per cent with Jørgensen's method. The limits of error ($P=0.99$) therefore, are 100 ± 5.44 and 100 ± 5.53 per cent respectively.

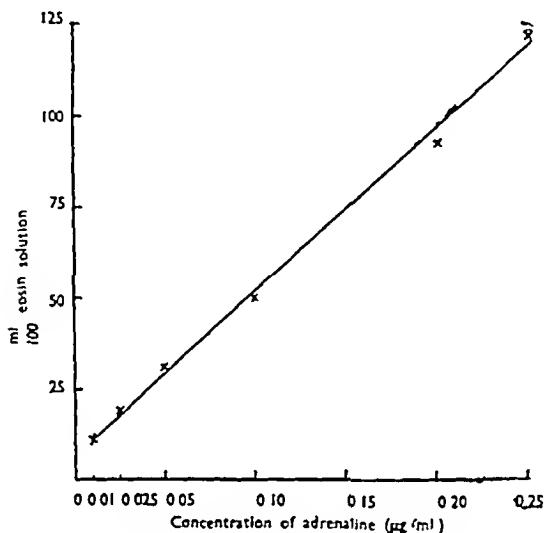


FIG 3—Calibration curve for fluorescence of alkali-treated adrenaline, using Jørgensen's eosin solution.

The effect of alkali on strong solutions of adrenaline

In order to measure the biological activity at different stages of the reaction, a more concentrated solution of adrenaline than previously used was treated with a weaker alkali, such as 1.5*N* Na_2CO_3 (as suggested by Shaw, 1941). The activity was determined using isolated rabbit gut, the perfused frog heart and perfused frog blood vessels and results are shown in Fig 4. A loss of 50 per cent was found after 15 min contact. Chemical estimations by Shaw's (1938) method with and without sodium sulphite and fluorimetric estimations were also carried out.

All the results are reduced to one scale and plotted on one graph (Fig 5). This graph suggests that the increase in reducing power is not due to the formation of the fluorescent substance (as Utevsky suggested), since maximal fluorescence only appeared after 20 min, and lasted only 5 min. There was a sudden increase in reducing power within 2 min and this increase (usually five-fold) was maintained for at least 30 min. When the sulphite was omitted from Shaw's test, the values followed the physiological activities very closely, but less than 10^{-6} adrenaline could not be detected by this procedure. There appears to be little or no connexion between loss of physiological activity (measuring rate of

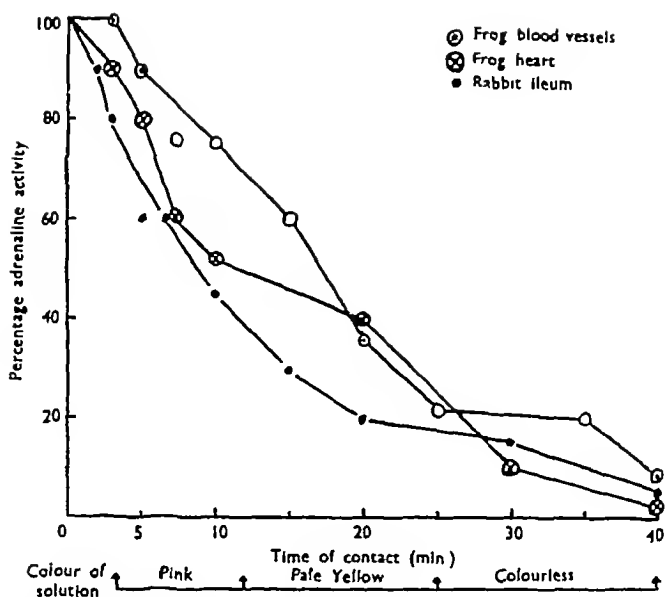


FIG 4—The physiological activity of adrenaline (10^{-5}) treated with 1/10 vol 1 $N Na_2CO_3$ for given times. The three methods of assay show fair agreement.

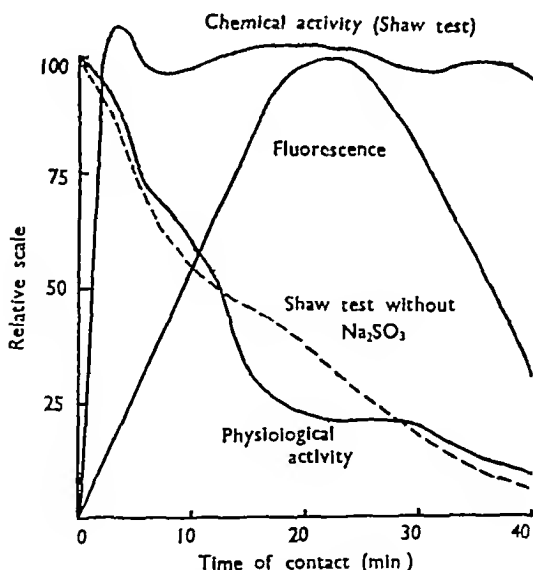


FIG 5—The chemical and physiological activities and the fluorescence of adrenaline solution (10^{-5}), when treated with 1 $N Na_2CO_3$ for given times.

destruction of adrenaline) and increase in reducing power or production of fluorescence

Sensitization of tissues to adrenaline by alkali-treated adrenaline

During tests of the physiological activity of alkali-treated adrenaline solutions on the perfused frog blood vessels, an interesting phenomenon was observed. In this preparation the two anterior venae cavae and the right aorta were ligatured, and Clark's Ringer solution from a Mariotte bottle was perfused via a special arterial cannula into the left aorta and collected from the posterior vena cava into a drop timer (Gaddum and Kwiatkowski, 1938). After about 4 hours' perfusion, during which time the preparation became much more sensitive to doses of adrenaline, consistent responses to 0.01–0.05 μ g. were obtained. The presence of oedema was not detrimental to the responses, which were graded according to the dosage (Fig. 6). The perfused blood vessels of female Winter frogs proved the most suitable test objects, moreover, they were relatively insensitive to histamine, acetylcholine and atropine in moderate doses.

A short-lived sensitization of the preparation to a subsequent dose of adrenaline was shown by the same dose of adrenaline previously treated with weak alkali (1.5N Na_2CO_3) for 20 to 25 min (Fig. 7). No such sensitization was detected in similar experiments when the adrenaline was treated with alkali for 15 min or less, or for more than 30 min. The time relations suggest that this sensitization is due to the fluorescent substance (Fig. 5).

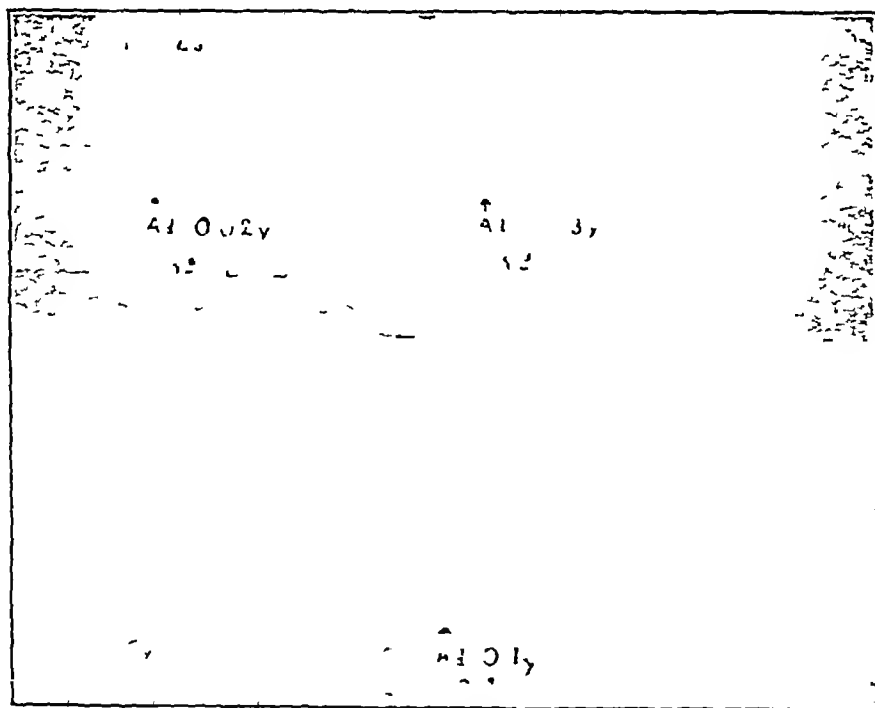


FIG 6—Record of outflow of perfused frog blood vessels, the dose-response tracing for adrenaline (Ad.)

because it was only short-lived. That it was not due to the volume of saline injected, the alkali used, or the adrenaline remaining active was shown by injecting these solutions alone just before the adrenaline dose. Jang (1940) has already reported a short-lived sensitization of adrenaline responses by tyramine, sympatol and adrenalone, but a prolonged sensitization

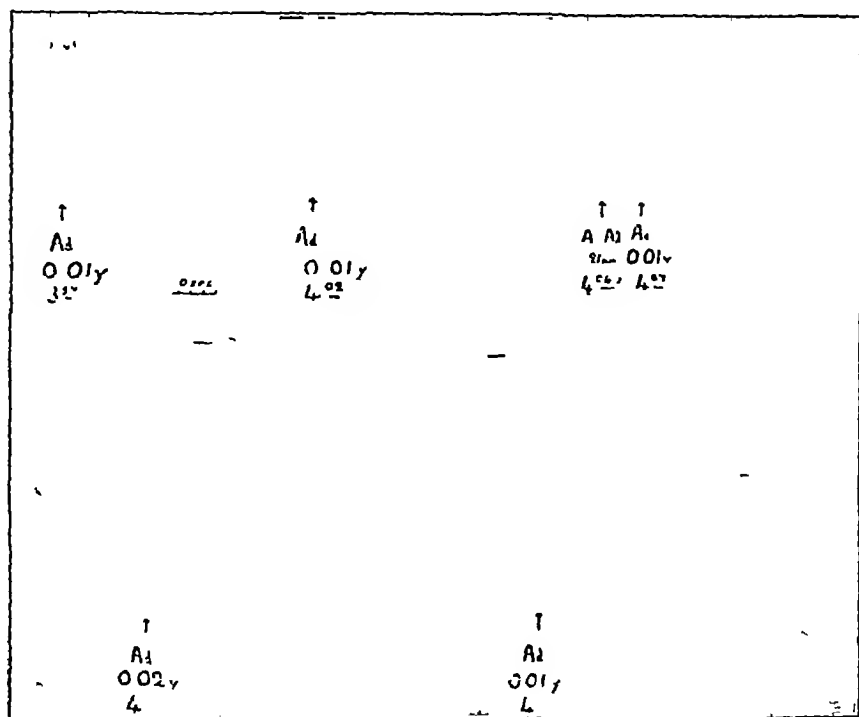


FIG 7—The effect of adrenaline, treated with 1.5N Na_2CO_3 for 21 min, and given 1/2 min before the standard dose of adrenaline (Ad 0.01 μE), on the outflow of the perfused frog blood vessels. Enhancement shown was not quite equal to that due to doubling the standard dose.

by phenylisopropylamines such as benzedrine and ephedrine, which are immune to amine oxidase. In the case of the alkali-treated adrenaline, there was some sensitization of the adrenaline response on the spinal cat, rabbit gut, and the Straub frog heart, but none on the perfused frog heart. On the cat's nictitating membrane depression of the adrenaline response sometimes occurred.

DISCUSSION

It is well known that adrenaline is unstable when dilutions are made in Tyrode or Locke solutions. The pink and red colours appear and with them is some fluorescence. Red oxidation products have formed the basis of many chemical methods of estimating adrenaline in solution, the common oxidizing

agents used being ferric chloride, potassium permanganate, persulphate, iodate or dichromate, and iodine. It must not be forgotten, however, that oxidation may occur in the side chain (amine oxidase of Blaschko, Richter, and Schlossmann, 1937).

The fluorescence of adrenaline in alkaline solution has been investigated and most of Utevsky's findings confirmed. That the effect of alkali on adrenaline should be oxidation followed by reduction is an unusual observation, but one explanation might be that a little adrenochrome is first formed and this is reduced at the same time as further adrenaline molecules are oxidized. The process may go on until all the initial adrenaline is oxidized. On the other hand, it is possible that leuco-adrenochrome is formed from adrenaline-quinone and is then oxidized to adrenochrome, in which case the fluorescent material would be another derivative of adrenochrome, giving both catechol and indol reactions.

It has been suggested that the fluorescent reaction is dependent upon a certain grouping such as $\equiv\text{C}-\text{CH}_2-\text{N}=(\text{Jörgensen, 1945})$, or even the phenylethylamine nucleus. Sympathomimetic amines similar to adrenaline, such as *p*-sympatol, epinine, noradrenaline, and "Dopa," produce the same green fluorescence in much stronger solutions (Gaddum and Schild, 1934), but tyramine, ephedrine, and benzedrine do not. Catechol itself produces traces of fluorescence in alkaline solution, so that the basis of the production is not clear. Nevertheless, the test is a specific one for adrenaline in concentrations of 10^{-5} and above, and, when used in conjunction with chemical and biological assays, it can form a good method for the identification of sympathomimetic amines.

From the results shown here, it is certain that the reducing agent in Shaw's test is not entirely composed of the fluorescent material. The brief preliminary treatment of adrenaline with *N*-NaOH for 2 min produced fluorescence, but the acid nature of the subsequent reagents soon removed any fluorescence before reduction commenced.

SUMMARY

1 Adrenaline in alkaline solution has been shown to produce the fluorescent material, which is probably leuco-adrenochrome, and the reactions leading to its production have been studied. This fluorescent test of Gaddum and Schild has been made of greater use by decreasing the concentration of alkali to $2N$ -NaOH and measuring the fluorescence in a simple fluorimeter.

2 A modified technique for perfusing frogs is described. This preparation is particularly suitable for the assay of adrenaline in tissue extracts since it is relatively insensitive to histamine, acetylcholine, and other substances in these solutions.

3 Fluorescent solutions obtained from adrenaline sensitized the perfused blood vessels of the frog to a subsequent dose of adrenaline, but had little action on cat preparations and rabbit gut.

I wish to express my indebtedness to Prof J H. Gaddum for laboratory facilities and valued suggestions

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THE ACTION OF ACETYLCHOLINE, ADRENALINE AND OTHER SUBSTANCES ON THE REFRACTORY PERIOD OF THE RABBIT AURICLE

BY

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In discussing the action of quinidine, veratrine, and strophanthin on the refractory period of cardiac muscle, Lewis and Drury (1926) pointed out that the effect on the absolute refractory period differed from that on the effective refractory period. The absolute refractory period is the length of time before the muscle will again respond to a stimulus, the effective refractory period is the time before the muscle will again transmit a propagated wave to any distance, and for most purposes it is this effective refractory period which is important. Thus Drury and Love (1926) found that quinidine actually shortened the absolute refractory period of the tortoise heart, but lengthened the effective refractory period, it is, of course, this action on the effective refractory period which makes quinidine of value to restore the normal rhythm in auricular fibrillation.

A method of measuring the effective refractory period in the auricular muscle of the rabbit heart has recently been described by Dawes (1946). Using this method to test different substances for their power to act as substitutes for quinidine and to prolong the refractory period of the auricular muscle, Dawes found that substances with several different pharmacological actions were effective. Thus the local anaesthetics amethocaine, cocaine, and procaine prolonged the refractory period, similarly, substances called spasmolytics, such as syntropan, trasentin, pethidine, and papaverine, also prolonged the refractory period. Dawes pointed out that procaine was not only a local anaesthetic, but had a spasmolytic action, shown by its power to reduce the stimulant action of acetylcholine on the rabbit intestine, and further that procaine diminished the action of acetylcholine in inhibiting the amplitude of contraction of the rabbit auricles beating spontaneously. Dawes states that it is remarkable that quinine, quinidine, and procaine antagonize the action of acetylcholine on many different types of tissue. He quotes Harvey (1939a, b), who showed that quinine and procaine reduced the action of acetylcholine on denervated skeletal muscle, he quotes Macgregor (1939), who showed that procaine reduced the pressor response

to acetylcholine in the atropinized cat, and also Stavraký (1932), who showed that quinine reduced the secretory action of acetylcholine on the salivary gland. It appears that many of the substances which prolong the refractory period have the general property of antagonizing the action of acetylcholine.

The question therefore arose what the action of acetylcholine itself on the refractory period of the auricular muscle might be, and the work described here concerns this action. Observations have recently been made by Wedd and Blair (1945) on the action of acetylcholine and adrenaline on the turtle ventricle. They studied the changes in the Q-T interval of the electrocardiogram. With concentrations of acetylcholine equal to 1 in 100,000 or higher, they observed slight shortening of the Q-T interval. In an earlier paper, Blair, Wedd and Young (1941) discussed the relation of the Q-T interval to other events in the cardiac cycle, and concluded that the Q-T interval coincided with the absolute refractory period. Wedd and Blair therefore consider that since acetylcholine and also carbaminoylecholine reduce the Q-T interval, they also reduce the absolute refractory period. In the paper by Drury and Love (1926) in which they distinguished between the refractory period as ordinarily measured and the absolute refractory period, it was shown that whereas quinine lengthened the refractory period as ordinarily measured, it diminished the absolute refractory period. It is therefore clear that no conclusion can be drawn about the effect of a drug upon the effective refractory period from its effect upon the absolute refractory period.

Dawes (1946) has already stated that adrenaline diminished the refractory period when it was tested on the isolated rabbit auricle. He did not, however, publish any figures relating the dose to the effect.

EXPERIMENTAL RESULTS

Acetylcholine—The preparation of the rabbit auricle was the same as described by Dawes (1946).

To test the effect of acetylcholine, 12 observations have been made in 4 preparations. As an example of the effect, before the addition of acetylcholine the auricle was able to follow stimulation at the rate of 260 per min, but not at the rate of 274 per min, each rate was applied twice with the same result. After 10 μ g acetylcholine had been added, the preparation followed stimulation first at 274 per min, then at 292 per min, but failed to follow at 314 per min, it then followed again at the rate 292 per min. Thus a concentration of 1 in 10 million shortened the refractory period by 12 per cent. Larger doses produced a greater effect, and the addition of 50 μ g enabled the auricle to follow a rate 40 per cent greater than the previous maximal rate. The different observations are given in Table I. In all the diminution of the effective refractory period is clearly seen. The relation between the percentage increase in the maximal rate at which the auricles would follow the imposed rhythm was found to be in linear

TABLE I
EFFECT OF ACETYLCHOLINE IN SHORTENING THE REFRACTORY PERIOD

Experiment No	Dose μ g	Rate per min at which auricle followed		Increase %
		before acetylcholine	in presence of acetylcholine	
1	2.5	292	338	15.7
	5.0	292	350	19.8
	10.0	292	363	24.0
2	50.0	284	400	40.9
	50.0	284	362	27.4
3	5.0	314	338	7.5
	10.0	314	362	15.2
	20.0	314	362	15.2
	40.0	314	388	24.0
	40.0	314	388	24.0
	80.0	314	414	32.0
4	10.0	260	292	12.3

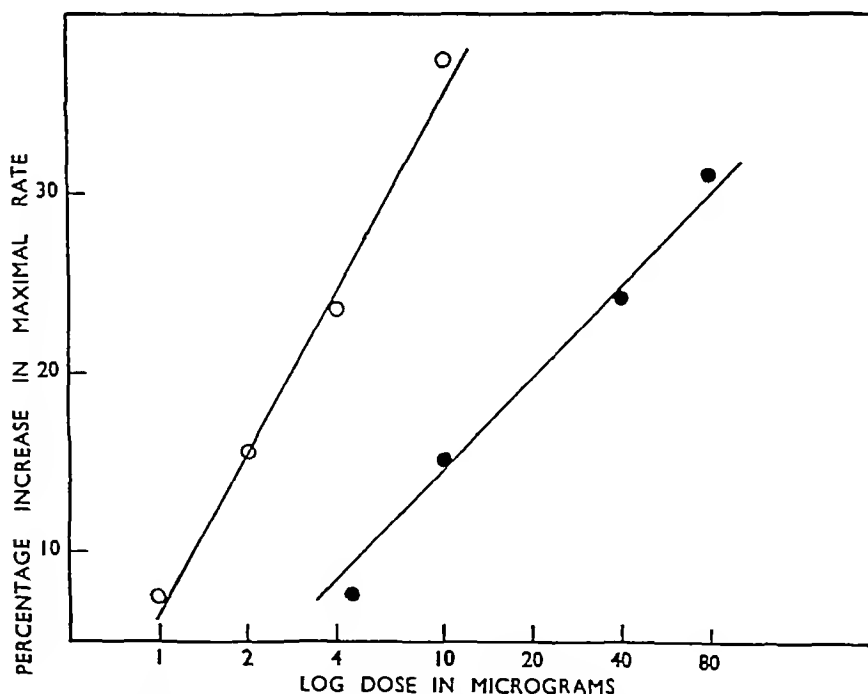


FIG 1—Ordinates, per cent increase in maximum rate at which the auricle responded to electrical stimuli. Abscissae, doses (bath of 100 ml.) on a logarithmic scale. The black circles show the effect of acetylcholine, the white circles show the effect of carbaminocholine.

proportion to the logarithm of the concentration of acetylcholine in one experiment. This is shown in Fig 1. The doses of acetylcholine used diminished the amplitude and the frequency of the spontaneous contractions of the auricles, though even the largest dose, which corresponded to a concentration of 1 in 1,250,000, did not cause complete arrest.

Carbaminoylcholine—Wedd and Blair (1945) found that carbaminoylcholine diminished the Q-T interval of the turtle ventricle, and I have found that, like

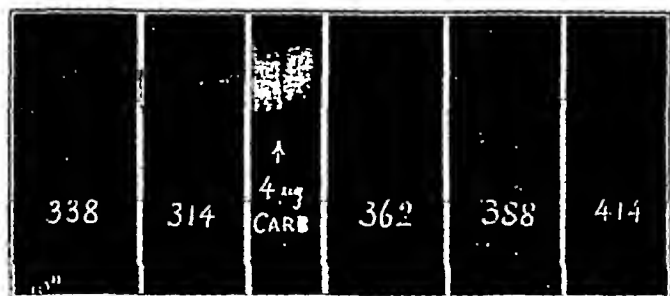


Fig 2—Isolated rabbit auricles driven electrically. On the left the auricles fail to follow 338 stimuli per min., but follow 314 per min. regularly. At the arrow 4 μ g. carbachol was added to the bath. This diminished the amplitude. The auricles then followed 362 and 388 stimuli per min., though at this high rate the amplitude was very small. At 414 per min. the auricles did not follow the stimuli.

acetylcholine, carbaminoylcholine increases the maximum rate at which the auricles will follow an applied stimulus. The spontaneous contractions of the auricles were arrested, but the responses to the electrical stimulation were more

TABLE II

EFFECT OF CARBAMINOYLCHOLINE IN SHORTENING THE REFRACTORY PERIOD

Experiment No	Dose μ g	Rate per min. at which auricle followed		Increase %
		before carbaminoylcholine	in presence of carbaminoylcholine	
2	50	292	430	47.2
5	25	252	304	20.6
	50	244	304	24.5
6	1	314	338	7.5
	2	314	362	15.5
	4	314	388	22.5
	8	314	430	37.7
	10	314	430	37.7
7	1	292	338	15.7
	2	292	338	15.7
	4	292	362	23.9
	8	292	388	32.7
	16	292	412	41.0

vigorous than usual. A concentration of 1 in 100 millions increased the maximal rate by 5.5 per cent, and concentrations in the neighbourhood of 1 in 10 millions increased the maximal rate by about 40 per cent. The observations are given in Table II, and the relation between the logarithm of the dose and the effect in one experiment is shown in Fig 1. An illustration of the effect on the auricles is given in Fig 2.

Adrenaline—To determine the effect of adrenaline on the maximal rate of stimulation was a more difficult matter. As already mentioned, Dawes found that adrenaline enabled the auricle to follow a higher rate than before and therefore shortened the refractory period. In most experiments I have confirmed this observation, but in others adrenaline had the opposite effect, at least at certain points (see Table III). Out of 28 observations, adrenaline enabled the

TABLE III
IMMEDIATE EFFECT OF ADRENALINE ON REFRACTORY PERIOD

Experiment No	Dose μ g.	Rate at which auricle followed			Immediate change %
		before adrenaline	in presence of adrenaline	after one washing	
8	10	274	292	260	6.2
	20	274	314	284	14.6
	40	274	378	292	19.0
	80	292	378	—	29.4
9	10	362	338	292	-6.6
	10	292	274	274	-6.1
	20	274	292	274	6.2
	40	274	314	314	14.6
	80	274	292	274	6.2
	160	274	314	274	14.6
	300	274	292	274	6.2
	600	274	274	—	0
10	20	260	274	260	5.3
	40	260	274	260	5.3
11	10	314	338	274	7.6
	10	314	338	274	7.6
	20	314	362	338	15.3
	20	314	338	292	7.6
	40	274	314	292	14.6
	80	292	314	292	7.5
12	10	274	292	244	6.2
	10	260	292	260	12.3
	20	274	338	260	23.3
	40	274	314	234	14.6
13	10	274	274	260	0
	20	274	274	244	0
	40	274	292	274	6.5
	40	274	292	260	6.5

auricle to follow at a higher rate in 23, but in the remaining 5 observations the rate was unaffected or reduced. As examples of reduction, the addition of 10 μg adrenaline to the bath of 100 ml reduced the rate from 362 to 338 per min and, in a second trial, from 292 to 274 per min. The changes in the maximum rate

TABLE IV
THE NUMBER OF TIMES A GIVEN CHANGE WAS OBSERVED

Dose of adrenaline $\mu\text{g. in 100 ml}$	Maximum rate			
	increased more than 10%	increased less than 10%	not changed	decreased
10	1	4	1	2
20	3	3	1	
40	4	3		
80	1	2		

caused by adrenaline are shown in Table IV, in which it is seen that as the concentration of adrenaline was increased more observations were made in which the maximum rate was increased. Thus when 10 μg was added to the bath, an

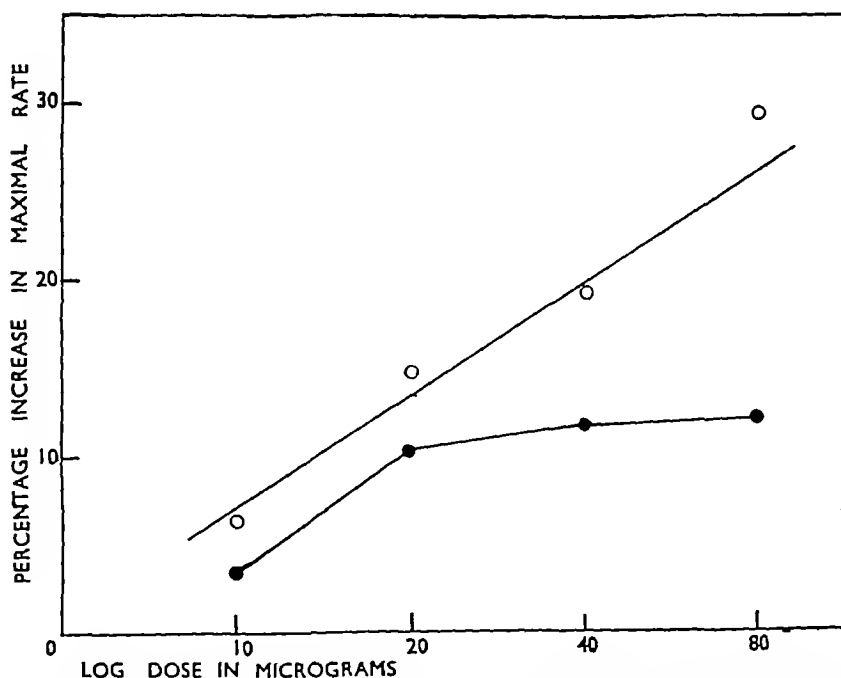


FIG 3—Ordinates and abscissae as in Fig. 1. The white circles show the results obtained with different concentrations of adrenaline in one experiment. The black circles are the mean results of all the observations made with adrenaline.

increase greater than 10 per cent was observed in 1 out of 8 trials, whereas when $40\text{ }\mu\text{g}$ was added such an increase was observed in 4 out of 7 trials. The mean of all observations is shown in Fig 3, in which the results of one exceptional experiment are also shown where the percentage increase in maximum rate was in linear relation to the logarithm of the dose. In some experiments the higher doses of adrenaline produced a smaller increase in maximal rate than lower doses.

A further curious observation was made that when adrenaline was added to the bath and then washed out, the maximum rate fell below the initial rate, thus the refractory period was first shortened by adding adrenaline, and then lengthened by washing it out. After further washing the rate returned by stages

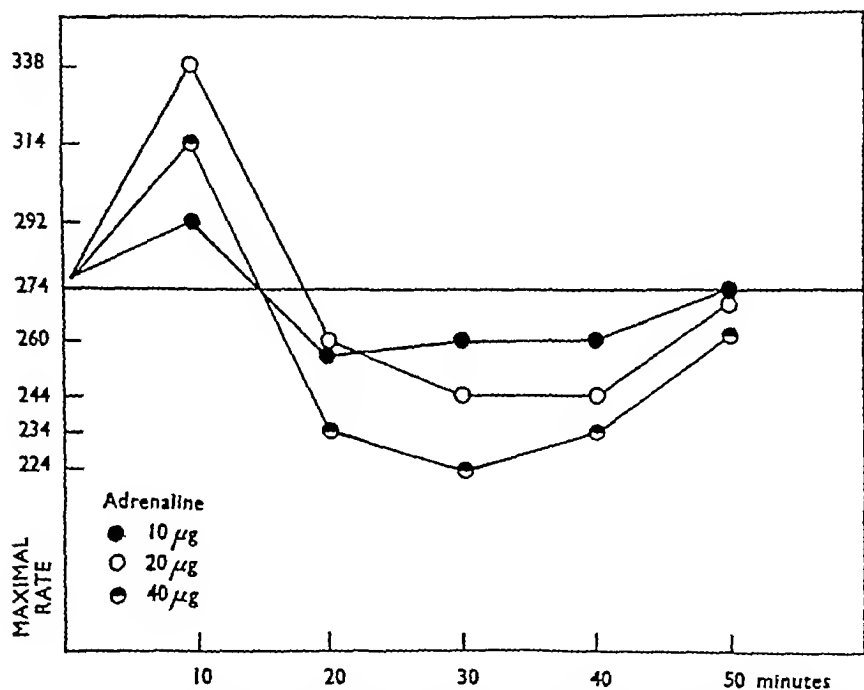


FIG 4—Ordinates maximum rate per min. at which the auricles responded to electrical stimuli. Abscissae time in minutes. The adrenaline added to the bath was in contact with the auricles only during the first 10 min., after washing out at 10 min. intervals, the maximum rate fell below the initial rate, and then gradually returned.

to the initial rate. Examples of this effect are given in Fig 4. This succession of changes caused by adrenaline was observed 9 times in 4 different experiments.

Nicotine—An action of nicotine (used as acid tartrate) on the isolated rabbit auricle was exerted when large doses were given. These produced an increase in amplitude as shown in Fig 5, no inhibitory effect was seen. Doses less than 0.8 mg (added to the bath of 100 ml) had no effect on the refractory period.



FIG 5 —Similar to Fig 2 This record is shown to demonstrate the effect of 1.6 mg. nicotine acid tartrate added at the arrow. Note the increase in amplitude similar to that produced by adrenaline, no inhibition is seen. After the addition of nicotine the auricles followed 274 stimuli per min., whereas before the addition the maximum rate was 260 per min.

but doses equal to this, or greater, usually increased the rate at which the auricles followed the applied stimulus, though the effect was small. In several observations, however, this was the initial effect only, and while the nicotine remained in the bath, the rate fell below the starting rate. Among the results given in Table V, there are examples of this double effect in each experiment. Thus in Exp 4, the addition of 3.0 mg caused an initial increase from 274 to 292 per

TABLE V
IMMEDIATE EFFECT OF NICOTINE ON THE REFRACTORY PERIOD

Experiment No	Dose mg	Rate at which auricle followed			Change (%)
		before nicotine	in presence of nicotine	after one washing	
4	0.4	274	274	274	0
	0.8	274	260	244	-5.1
	1.6	260	274	274	+5.3
	3.0	274	292		+6.5
			224	234	-18.2
	1.6	274	244	260	-10.6
13	0.08	274	274	266	0
	0.2	266	266	266	0
	0.8	266	274		+3.0
			238	266	-10.5
	1.6	266	284		+6.7
			238	266	-10.5
	3.0	266	304		+14.2
			252		-5.2
14	0.8	314	338	292	+7.6
	1.6	292	326		+11.6
			292	314	-7.5
	3.0	292	314		+7.5
			244	314	-16.4
	1.6	314	338	292	+7.6

min., followed by a decrease to 224 per min before washing out. The two phases of the nicotine action appeared to correspond to a stimulant stage and a paralysing stage. Thus, after testing the effect of 30 mg., a retrial of the effect of 16 mg produced a fall in the maximal rate, whereas earlier it produced a rise.

Prostigmine—The effect of prostigmine was tested in doses of 1 mg and 2 mg. These amounts produced either no effect or slight increases of 5 or 6 per cent in the maximal rate, they greatly augmented the effects of acetylcholine and of nicotine, as shown in Table VI. Whereas 200 μ g of acetylcholine alone increased the rate by 39 per cent, in the presence of 10 mg prostigmine 10 μ g of acetylcholine increased the maximum rate by 57 per cent.

TABLE VI
EFFECTS IN THE PRESENCE OF PROSTIGMINE

Substance	Dose μ g	Rate at which auricle followed			Change %	
		before drug	in presence of drug	after one washing	(a)	(a) to (b)
Exp 15						
acetylcholine	200	260	362 (a)	292	39	
prostigmine	1,000	260	274 (a)		5	
acetylcholine	10		430 (b)	260		57
prostigmine	2,000	260	260 (a)		0	
acetylcholine	20		388 (b)	292		49
nicotine	1,600	260	388 (a)	260	49	
acetylcholine	10	260	388 (a)	244	49	
Exp 16						
prostigmine	1,000	274	292 (a)		6	
acetylcholine	100		388 (b)	314		33
prostigmine	2,000	292	292 (a)		0	
acetylcholine	200		430 (b)	274		47
prostigmine	2,000	274	292 (a)		6	
nicotine	1,500		362 (b)	274		24
prostigmine	2,000	274	292 (a)		6	
nicotine	3,000		362 (b)	274		24
prostigmine	2,000	274	292 (a)		6	
nicotine	1,500		292 (b)	274		0
nicotine	1,500	274	274 (a)		0	

The increases in maximal rate produced by nicotine and recorded in Table V were often small, in the presence of prostigmine, however, they were very large, thus 16 mg nicotine added to the auricle when some prostigmine effect remained (after washing out 2 mg prostigmine), increased the maximum rate by 49 per cent.

An attempt was made to see if large doses of acetylcholine, added in the presence of prostigmine, would depress the maximum rate. No such depression was observed as shown in the second part of Table VI, though the repetition of large doses of nicotine led to disappearance of their action.

Eserine —Eserine acted similarly to prostigmine. Doses of 200 μ g were themselves without effect, but in their presence the action of acetylcholine was increased, thus 1, 2, and 4 μ g acetylcholine produced increases of 14.5, 20, and 39 per cent in the maximal rate. Eserine likewise increased the action of nicotine, in the presence of 200 μ g eserine, nicotine in a dose of 0.1 mg increased the maximum rate by 14.5 per cent, but larger doses of 0.2 mg and 0.4 mg nicotine tested afterwards had less action, the increase being 6.5 per cent for each dose.

Atropine —When atropine in a dose of 1 mg was added to a fresh preparation there was, in each of three experiments, a slight diminution in the maximum rate. After this addition, acetylcholine failed to cause the usual increase. In some experiments it appeared that after atropine, the action of adrenaline disappeared, but this was not always so, and in view of the variation in the response to adrenaline, it was not possible to conclude that atropine affected its action, nor did atropine modify the action of nicotine.

DISCUSSION

The results obtained in this work are in line with the points brought out by Dawes concerning the substances which resemble quinidine in its action on the electrically driven auricle. Dawes found that local anaesthetics and spasmolytics have a quinidine-like action and that they share the property of antagonizing the action of acetylcholine in stimulating the isolated intestine of the rabbit and in inhibiting the spontaneous contractions of the isolated rabbit auricles. He pointed out that on skeletal muscle also the action of a local anaesthetic like procaine resembles that of quinine. From Dawes's observations the conclusion can therefore be drawn that substances which prolong the refractory period of cardiac muscle are, in general, antagonists of acetylcholine. The inference from this conclusion is that acetylcholine, and substances with a similar action, should shorten the refractory period. The observations described in this paper show that acetylcholine has this action and that carbaminoylcholine has the same action. The effect of acetylcholine is increased in the presence of eserine or prostigmine, and it is abolished in the presence of atropine. The action of adrenaline is similar in direction to that of acetylcholine, but as the dose of adrenaline is increased there is little increase in effect. With acetylcholine there is a linear relation between log dose and the increase in the maximum rate at which auricles can be driven over a considerable range. With adrenaline this is not so, with increasing doses of adrenaline the increase in maximum rate is much less, and soon reaches a point beyond which no further increase is observed.

Since the diverse substances which have a quinidine-like action on the auricles have the common property of antagonizing the action of acetylcholine, the suggestion arises that the transmission of the impulse in cardiac muscle may be effected by a mechanism in which acetylcholine is a key substance and in which

the rate of transmission is governed by the rate of formation of acetylcholine. Abdon and Hammarckjold (1944) have demonstrated that rabbit hearts, among other tissues, contain a precursor from which acetylcholine can be liberated, so that such a mechanism is a possibility. The application of acetylcholine to the isolated auricle might then be supposed to facilitate the working of the mechanism by providing acetylcholine ready made. The action of adrenaline could be regarded as an action in which it potentiated the effect of acetylcholine, the extent of the potentiation being limited. Other examples of the potentiation of acetylcholine by adrenaline have been described by Bulbring and Burn (for references, see Burn, 1945). The action of acetylcholine in shortening the refractory period is abolished by atropine, and if acetylcholine is concerned in the transmission of the normal process of excitation, it might be expected that atropine would be a potent substance in prolonging the refractory period. Several examples, however, are known where a normal process mediated by acetylcholine is not appreciably affected by atropine, although atropine abolishes the action of acetylcholine when externally applied. When, however, such a normal process is exaggerated, then atropine removes the exaggeration. Novoa Santos in his textbook of general pathology (1934) describes how auricular fibrillation in a proportion of patients is arrested by atropine, if it is supposed that in these cases the fibrillation is due to excessive formation of acetylcholine, and that atropine antagonizes the action of this excess, we can then understand how atropine acts. In a similar fashion, we can understand how atropine removes the tremors of paralysis agitans without affecting ordinary voluntary movements. Ordinary movements appear to require the mediation of acetylcholine at the synapses, spinal reflexes are not, however, modified by atropine. The effect of accumulations of acetylcholine in the spinal cord, whether inhibiting or stimulating is however, abolished by atropine (Bulbring and Burn 1941).

SUMMARY

- 1 Observations have been made on the isolated auricles of the rabbit driven electrically. Acetylcholine and carbaminoylecholine increase the maximum rate at which the auricles will follow the stimulation applied.
- 2 The relation between the percentage increase in maximum rate and the logarithm of the dose is linear for both these substances.
- 3 The effect of acetylcholine is increased by eserine or prostigmine and abolished by atropine.
- 4 Adrenaline has some effect in increasing the maximum rate of stimulation, but this effect increases only slightly with increase of dose.
- 5 Nicotine has an initial acetylcholine-like action in large doses, which is followed by the opposite effect probably owing to partial paralysis.
- 6 The mechanism of these changes is discussed.

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THE INHALATION OF HEXOESTROL

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The word gynaecomastia was first used by Galen to describe an enlargement of the male breast (Karsner, 1946). Such a condition may occur after the administration of natural oestrogens (Dunn, 1935). It occurred, with increased pigmentation of the areola and nipple, in a young man treated for acne with injections of oestradiol benzoate (Foss, 1940). Dunn (1940, 1941) reported gynaecomastia after 5 mg of stilboestrol daily by mouth for about 2-3 months in sexual criminals. Reports have since been made of similar effects after synthetic oestrogen therapy for prostatic carcinoma (Moore, Wattenburg, and Rose, 1945) and for acne (Simon, 1945).

Scarff and Smith (1942) described two cases occurring in a factory where the highly potent synthetic oestrogen stilboestrol was manufactured in quantity, even when stringent measures of protection were adopted. Occupational gynaecomastia then became a recognized entity, and Fitzsimmons (1944) has described the clinical findings in 20 cases, together with the routes of absorption and the preventive measures taken.

In the early months of 1942 we were interested in this industrial hazard, which naturally was causing a little concern among the workers as in addition to the gynaecomastia there was the real risk of impotence and testicular atrophy if only of a temporary nature (Dunn, 1940, 1941) and possibly of malignant change occurring in breasts showing "a dangerous degree of epithelial proliferation" (Scarff and Smith, 1942).

After ingestion of natural oestrogens, much of the activity of a given dose is lost by detoxication in the liver, and it was shown by Biskind and Mark (1939) that a pellet of oestrone implanted in the spleen and thus absorbed via the liver was considerably less efficient in producing positive vaginal smears in castrated female rats than a pellet of the same size and shape implanted subcutaneously. Stilboestrol and other synthetic oestrogens probably behave somewhat differently and are effective by the mouth.

Workers in factories where synthetic oestrogens are made must therefore be protected against the risk of swallowing small quantities or of absorbing them by the skin. They are also liable to be exposed to the inhalation of fine dusts contaminated with oestrogens. The work described below was undertaken in

order to assess the effects of such exposure and to compare the effects with those of similar doses by the mouth

METHODS

Experiments have been made with male human volunteers and with guinea-pigs. With men no special methods were used, but with guinea-pigs the effect was measured.

Male guinea-pigs show well marked growth of the nipples with development of mammary tissue after a total dose of 0.2 mg. of hexoestrol has been injected subcutaneously every other day for 20 days (Dodds, 1939). In the experiments described here, light-coloured or white guinea-pigs weighing 300–700 gm were clipped around the nipples, and were then anaesthetized with ether and photographed under constant conditions. The right nipple was held lightly resting on a fixed and rigid millimetre scale, with white divisions on a black background. The source of light was constant, and the same aperture and the same exposure were used each time. These photographs were all enlarged 7 times, and the length of the nipple and the diameter through the centre of its length were measured by comparison with the photographed scale. The volume of the nipple was then calculated on the assumption that it was a cylinder, the tapering at the tip was roughly balanced by the broadness of the base. The volume of the nipple was measured in this way before treatment and 14 days later, and the percentage increase in volume was calculated.

Hexoestrol was administered in solution in arachis oil, either by stomach tube or by inhalation of the spray of a Collison (1935) inhaler. The spray was directed down a wide glass tube with a large hole in the side of it partly closed by a rubber diaphragm, through which the guinea pig's nose was poked. The dose of hexoestrol inhaled by the guinea pigs was roughly estimated in the following way. The output of the spray was 9.4 litres of air and 120 mg of oil per minute, so that the concentration of the oil in the cloud breathed by the guinea-pig was about 12.8 mg/litre. It was assumed that the guinea pigs, which weighed about 500 g, breathed 250 c.c. of air or 3 mg of oil per minute (Gaddum 1944).

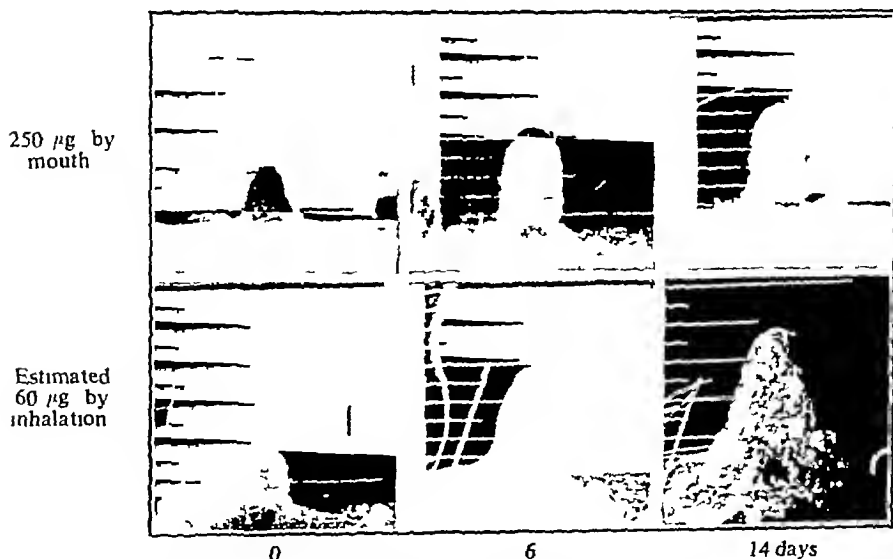


FIG 1—Nipples of guinea pigs before and after a single dose of hexoestrol in arachis oil
Scale mm and 0.5 mm

The dose of hexoestrol was then calculated from the known concentration of the drug in the oil and the duration of the exposure. The assumptions involved make these estimates of the dose unreliable. A small amount of hexoestrol was deposited on the walls of the apparatus and the actual dose inhaled was probably less than the estimate, but it seems likely that it was more than 50 per cent of the estimate.

RESULTS

Guinea-pigs—In two experiments the effect by the mouth was compared directly with the effect by inhalation. Guinea-pigs which inhaled hexoestrol in the first experiment were not photographed individually before the treatment,

TABLE
GUINEA-PIGS EFFECTS OF HEXOESTROL ON NIPPLE VOLUME

Weight gm	Approx dose µg.	Time of exposure min.	Normal nipples			After 14 days			% mc	Remarks
			Length mm.	Width mm.	Volume cu.mm.	Length mm.	Width mm	Volume cu.mm		
EXPERIMENT 1										
By mouth using 0.1% and 0.01% solution in Arachis Oil.										
570	50	—	1.7	1.2	1.92	2.9	1.6	5.8	202	
710	100	—	1.8	1.1	1.69	3.4	1.6	6.8	243	
520	250	—	1.5	1.2	1.70	3.3	1.8	8.4	390	
600	500	—	1.7	1.1	1.60	4.0	1.7	9.0	460	
550	750	—	2.2	1.2	2.49	4.8	2.0	15.0	500	
660	1000	—	1.7	1.0	1.34	4.0	1.9	11.2	735	
By inhalation using 1% solution in Arachis Oil										
355	30	1				3.1	2.4	14	610	
305						—	—	—	—	Died 6 days
305	60	2			1.92	4.3	2.5	21	1000	
400					(Mean	3.9	2.8	24	1150	
390	120	4			of 26)	3.3	2.0	10.4	440	
330						4.0	2.2	15.2	690	
EXPERIMENT 2										
By mouth using a 0.001% solution in Arachis Oil										
520	2.5	—	1.8	1.0	1.34	1.8	1.0	1.34	0	
650		—	2.6	1.3	3.4	2.5	1.2	2.8	-18	
550	5	—	2.4	1.2	2.7	2.4	1.1	2.25	-17	
500		—	1.7	1.0	1.33	—	—	—	—	Died 5 days
620	10	—	2.2	1.1	2.1	2.2	1.2	2.5	19	
530		—	2.0	1.1	1.9	2.1	1.1	2.0	5.2	
600	25	—	2.0	1.1	1.9	2.3	1.2	2.6	37	
600		—	2.0	1.1	1.9	2.1	1.2	2.38	25	
700	40	—	2.5	1.5	4.4	—	—	—	—	Died 11 days
520		—	1.9	0.9	1.2	1.9	1.1	1.8	50	
By inhalation using a 0.1% solution in Arachis Oil										
520	0	0	1.7	1.2	1.9	1.7	1.1	1.6	-16	
530		—	2.2	1.5	3.9	—	—	—	—	Died 16 days
600	3	1	2.0	1.4	3.1	2.2	1.4	3.38	11	
480		—	2.2	1.4	3.4	2.5	1.4	3.85	13	
590	6	2	1.8	1.0	1.4	2.8	1.4	4.15	195	
540		—	1.9	1.3	2.5	2.1	1.6	4.2	63	
500	12	4	2.2	1.2	2.5	4.0	1.8	10.1	300	
420		—	1.4	0.9	0.88	2.5	1.5	4.4	250	Died
—	24	8	1.2	0.9	0.75	—	—	—	—	31 days
650		—	1.5	1.3	1.98	2.4	1.6	4.8	143	Died 16 hr

and the percentage increase in volume was calculated by comparing the volumes after treatment with the average volume of 26 normal nipples, belonging to guinea-pigs of about the same weight. With this exception the volume of each nipple was estimated before and after treatment. The results of these experiments are shown in the Table and Figures

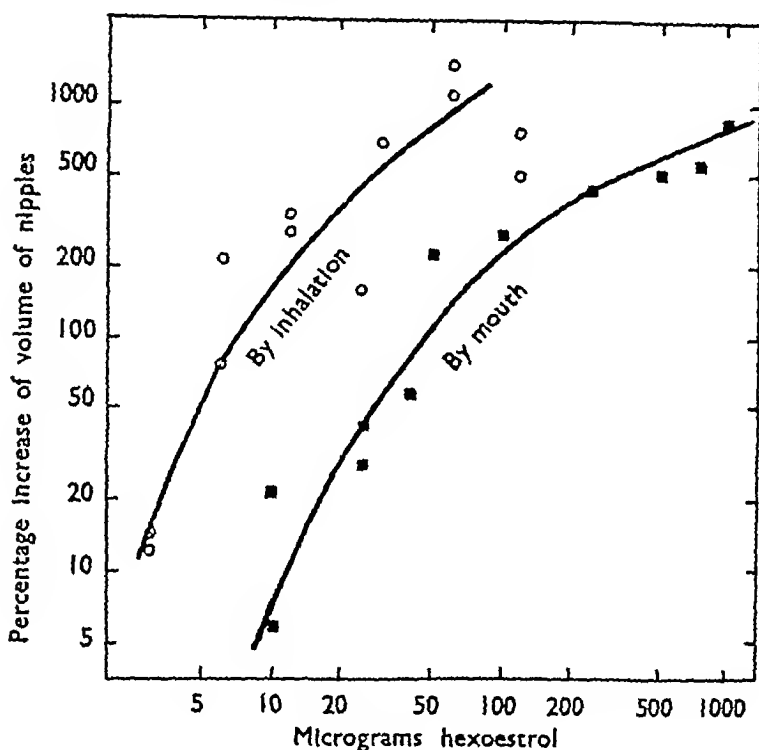


FIG. 2—Effects of hexoestrol given to guinea-pigs by inhalation (circles) and by the mouth (squares). Logarithmic scales. Horizontally, the estimated dose in micrograms per animal. Vertically, the percentage increase of the volume of the nipples after 14 days.

The deaths recorded in the table were probably not due to hexoestrol. Deaths occurred, among normal guinea-pigs in the stock, rather frequently during this period. The figures are erratic owing to the normal variation between guinea-pigs and the smallness of the numbers used, but they suffice to show approximately the threshold doses for a single dose by the two methods of administration. Hexoestrol in oil was 5–10 times as effective by inhalation as it was by the mouth.

Man—A series of 7 male observers were given single doses of 15–50 mg of stilboestrol by mouth without any effect on the nipples or breasts at all. Some of these men, consisting of professors, doctors, and laboratory assistants, noticed

loss of appetite, slight headache, and malaise. One doctor took 55 mg in 4 days without any effect at all on the nipples. A laboratory assistant swallowed 5 mg. a day for 17 days. He noticed some indigestion and an empty feeling in the epigastrium, and at the end of this time he said that he was conscious of his nipples being rather sensitive and he felt the pressure of his pyjamas on them. On examination the areola were bright pink and there was slight enlargement of the nipples, but there was no thickening of the breast tissue beneath. He then stopped taking tablets, as he felt a little anxious.

DISCUSSION

The experiments on guinea-pigs show that hexoestrol in arachis oil is very effective by inhalation. The effect shown in Fig 1 was due to exposure for only 2 min altogether to a concentration of hexoestrol estimated as 128 mg. per cubic metre. The curves shown in Fig 2 suggest that the dose for a given effect by inhalation is only about 1/5th of the dose for the same effect by oral administration. If the conclusions given above about the error of the estimate of the amount inhaled are true, then the dose by inhalation lies between 1/5th and 1/10th of the dose by oral administration. It is possible that the large effects observed after inhalation were due to the formation of a depot of arachis oil in the lungs from which the hexoestrol was slowly absorbed.

The nipples of man appear to be less sensitive than those of the guinea-pig when hexoestrol is taken by the mouth and when doses are calculated in mg per kg. Thus a dose of 0.25 mg or 0.5 mg per kg, given by the mouth to a male guinea-pig, increased the volume of the nipple five times. The length of the nipple was more than doubled and the general effect was definite, but not maximal. On the other hand, a dose of 50 mg, or nearly 1 mg per kg, had no observed action on man. The drug is probably more effective when given in repeated small doses, but it is apparent from Dunn's experiment and from those recorded here that 5 mg per day is only effective if continued for several weeks.

There is no quantitative information available about the effects of inhalation by man, and calculations about this must be based on indirect evidence. A dose of 5 mg of hexoestrol per day by the mouth is likely to lead to troublesome effects if continued. Judging by the experiments with guinea-pigs, this would be equivalent to between 0.5 and 1 mg per day by inhalation. If a man inhales 15 litres per minute, he will inhale 72 cu.m in a working day of 8 hours. It would therefore be expected that a concentration of 0.5-1 mg in 72 cu.m, or 0.07-0.14 mg per cu.m, would be harmful after several weeks if exposure were continuous for 8 hours a day. Fitzsimmons (1944) observed a concentration of 0.2 mg of stilboestrol in 300 cu.ft, or 0.023 mg per cu.m, on one occasion in the Glaxo laboratory. Our calculations suggest that such concentrations of hexoestrol would not be immediately harmful but are near the dangerous limit if maintained for several weeks.

Fitzsimmons found that the vaginal smears of ovariectomized mice became positive when they were kept for 3 days in various parts of the factory where stilboestrol was being prepared, even when careful precautions were taken to prevent the dispersal of the oestrogen in the air. An alternative biological test is now available. If male guinea-pigs were kept in factories where synthetic oestrogens are made, their nipples could be inspected, and if necessary measured, at regular intervals and so used as an index of danger. This would be simpler than the vaginal smear technique, though presumably less sensitive. It is probable, however, that the smear test is too sensitive and becomes positive when there is no danger. There is reason to believe that the guinea-pig's nipple will provide an indicator of oestrogens in the air which is sensitive enough for practical purposes. The guinea-pig is more sensitive than man to hexoestrol as judged by the oral dose per kg of body weight, he inhales more air, and thus more oestrogen, per kg of body weight and is thus likely to be relatively even more sensitive to clouds than to oral administration. If a man and a guinea-pig were exposed daily to clouds of oestrogen together, the guinea-pig should therefore be affected first and give adequate warning to the man.

The results also suggest a method for the therapeutic administration of oestrogens. They have been given by ingestion, injection, inunction, and implantation. They might also be given by inhalation, but it would probably be difficult to control the dosage and there would be no obvious compensating advantages in this method of administration.

SUMMARY

1 A solution of hexoestrol in arachis oil was administered to male guinea-pigs and the effect on the nipples was photographed and measured.

2 A given dose had more effect when given by inhalation than when given by the mouth.

3 It is suggested that male guinea-pigs should be kept in places where synthetic oestrogens are made, to give warning of any failure of the measures taken to protect the workers from the effects of the oestrogens.

We are indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish this paper.

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2-*p*-AMINOBENZENESULPHONAMIDO-4,6-DIMETHOXYPYRIMIDINE EXPERIMENTAL EVALUATION

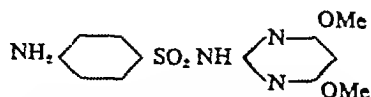
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The introduction of an α -pyridyl residue into the molecule of sulphanilamide by Ewins and Phillips (1937) was a major advance in the development of sulphanilamide therapy, since not only was there produced a marked improvement in intrinsic antibacterial activity, but the new substance, sulphapyridine, was the starting point for an extensive research on the preparation of sulphanilamide drugs derived from other heterocyclic systems. The pyrimidine ring system has been particularly fruitful and at least three drugs derived from this nucleus are in common use—sulphadiazine, sulphamerazine, and sulphamezathine. They are characterized by antibacterial activity of a high order against a wide range of organisms, by rapid and efficient absorption from the gastrointestinal tract, and by a degree of persistence in the blood which provides economy in use. The work of Bell and Roblin (1942) suggests that the discovery of sulphanilamide drugs possessing a higher intrinsic antibacterial activity than that exhibited by the sulphapyrimidine group is becoming more remote. Any improvement in sulphanilamide therapy must therefore arise from research devoted to the production of drugs exhibiting reduced toxicity and enhanced persistence in the blood. We have interested ourselves for some time particularly in the latter aspect, and have found this property to be outstanding in sulphanilamides derived from 2-amino-4,6-dialkoxypyrimidines. A series of such compounds has been prepared (Rose and Tuey, 1946) and we propose to publish in due course detailed pharmacological and bacteriological findings on these substances as a class. This memoir is concerned with the parent compound 2-*p*-aminobenzenesulphonamido-4,6-dimethoxypyrimidine (sulphadimethoxypyrimidine), which is the most effective of the many homologues which have been examined.



While this paper was in preparation our attention was drawn to the researches of van Dyke, Tupikova, Chow, and Walker (1945), who, in the course of an

extensive study of sulphapyrimidines, have examined some of the 4-6-dialkoxy derivatives with which we have been concerned. In the main, these authors confirm our findings.

We describe the pharmacology and *in vitro* antibacterial properties of sulphadimethoxypyrimidine, the combination of these factors in therapeutic activity, the toxicity and the physico-chemical properties. A note on the unusual properties of the acetyl derivative of sulphadimethoxypyrimidine is included.

The clinical activity and pharmacology of the drug are now being investigated at Crumpsall Hospital, Manchester, and will be reported later by the workers concerned.

PHARMACOLOGY

The blood concentrations attained by sulphadimethoxypyrimidine have been examined in the mouse, rat, rabbit, dog, chick, sheep and calf. For data on the last two species we are indebted to Mr J Francis, of our Veterinary Research Laboratories.

In the mouse—Absorption was examined by the standard technique already described (Rose and Spinks, 1946). Three mice received 250 mg./kg. by stomach tube as a 1 g/100 ml. solution of the sodium salt, and the drug was estimated at intervals in pooled tail blood by the method of Rose and Bevan (1944). In a series of 14 analyses of 0.05 ml samples of blood containing 2 to 20 mg./100 ml, the mean recovery was 98.5 per cent \pm standard deviation 6.95 per cent, limit of error for a probability level of 0.05, ± 4.00 per cent.

The single experiment was repeated 22 times, so that 66 animals contributed to the mean results (Table I), in Fig. 1 the mean absorption curve is compared with that of sulphamerazine.

TABLE I
BLOOD CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE
250 mg./kg. in groups of 3 mice

Route of administration	Mean blood concentrations in mg/100 ml after								
	20 min	40 min	1 hr	1.5 hr	2.5 hr	3.5 hr	5 hr	7 hr	24 hr
Oral (22 expts)	11.6	12.7	13.2	14.1	13.8	14.4	14.3	14.0	9.4
Intraperitoneal (13 expts)	17.7	21.5	21.1	20.2	20.4	19.3	19.1	17.0	9.4
Subcutaneous (6 expts)	14.1	19.1	18.8	19.9	17.0	20.6	20.3	18.6	7.7

In an earlier paper (Rose and Spinks, 1946) the expression C7, which is the time required for the blood concentration to fall from that attained 7 hours after dosing to two-thirds of this figure, was suggested as a convenient means of defining the rate of disappearance of a sulphonamide from the blood. Sulphadimethoxypyrimidine is clearly more persistent than sulphamerazine, the values

of C7 taken from the mean curves being 16.2 and 7.3 hours respectively. By obtaining the characteristic values from individual curves (Rose and Spinks, 1946) and submitting the results to statistical analysis, the difference is readily shown to be decisive ($P < 0.01$). Sulphadimethoxypyrimidine is thus 2.2 times as persistent as sulphamerazine in mice, the term persistence being taken to mean the retention of free drug in the blood. The new drug reaches fairly high concentrations, the mean of the maximum concentrations from individual curves is 15.3 ± 1.25 mg/100 ml, which is higher than the corresponding figure for

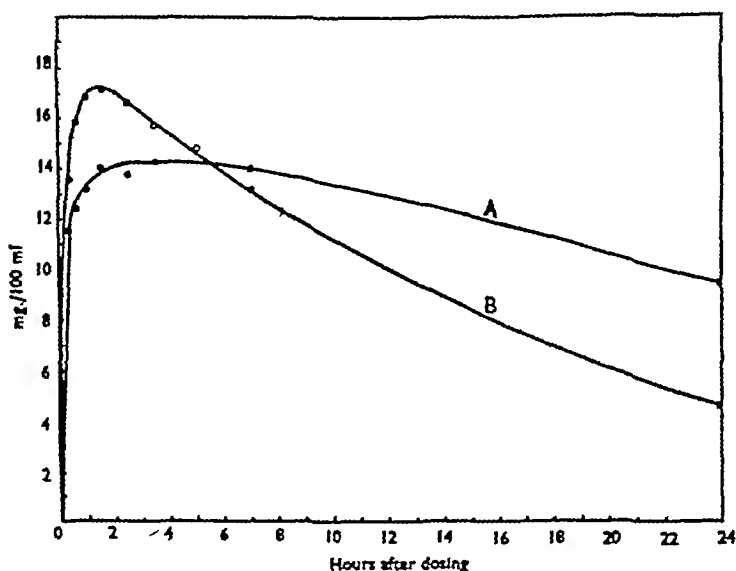


FIG 1—Blood concentrations in mice of sulphadimethoxypyrimidine (A) and sulphamerazine (B), following the administration of 250 mg/kg. orally

sulphanilamide, sulphapyridine, or sulphathiazole, although lower than that for sulphamezathine, sulphamerazine, or sulphadiazine. Sulphadimethoxypyrimidine is rather slowly absorbed, the time at which the maximum blood concentration is attained, taken from the mean curve, being 220 minutes. Using the method of statistical analysis already described (Rose and Spinks, 1946), the drug can be shown to be significantly more slowly absorbed than any of the other three sulphyrimidines.

The blood concentrations attained by the drug following intraperitoneal and subcutaneous administration of 250 mg/kg. to mice are shown in Table I. From these it must be concluded that the drug is more rapidly and more completely absorbed by these routes than when given orally. Persistence in the blood is again marked, indeed there is no significant difference in this respect between the intraperitoneal and oral routes. The mean maximum blood concentration

of individual intraperitoneal curves (20.7 mg/100 ml) is decisively higher than that of individual oral curves (15.3 mg/100 ml), and the difference between the mean times of attaining these maximum values is also decisive. Owing to the difficulty of drawing some of the individual subcutaneous curves, no statistical comparison has been attempted in this case.

Sulphadimethoxypyrimidine can be detected in the blood of mice for several days after the administration of a single oral dose of 250 mg./kg. Blood concentrations recorded in a typical experiment with three mice were

Time (hr)	2	22	27	42	46	66	71	73	89	139
mg./100 ml	13.2	13.85	13.4	5.0	5.3	4.0	4.2	1.2	0.25	0.14

The maintenance of high concentrations for so extended a time suggested that repeated dosing might have a cumulative effect, giving dangerously high concentrations. This possibility was examined by administering two oral doses of 250 mg/kg at an interval of

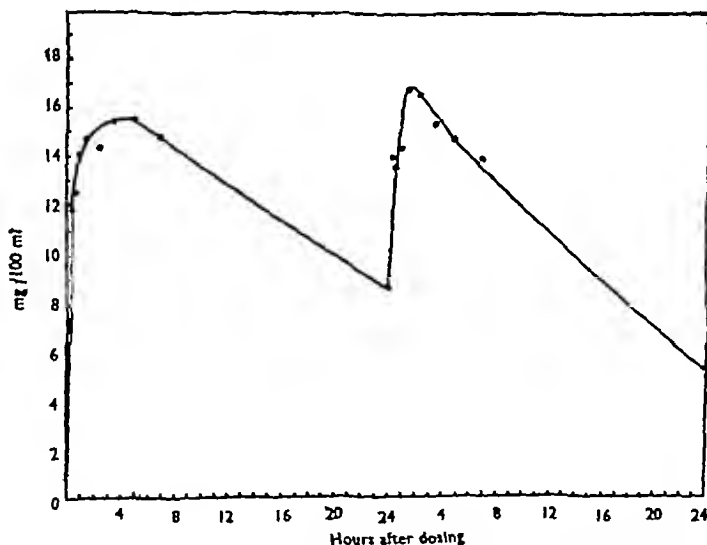


FIG 2.—Blood concentrations of sulphadimethoxypyrimidine following the administration to mice of two oral doses of 250 mg./kg at an interval of 24 hours

24 hours to a group of three mice and estimating the concentration of free drug in the blood at suitable intervals after each dose. The experiment was repeated ten times, with the results shown in Fig. 2. Clearly there is no marked rise in maximum blood level on such repeated dosing. This has been confirmed by statistical treatment, which shows that there is no significant difference between the mean maximum concentrations, but there are decisive differences between the mean times at which these maxima were attained and between the respective rates of disappearance of the drug from the blood stream. We have no explanation to offer for the greater rapidity with which the second dose is absorbed but it may be that this phenomenon is associated in some way with the lower persistence

Tissue concentrations in mice have been estimated following the usual dose of 250 mg/kg., three mice being used for each point recorded in Table II. In Table III the corresponding values for sulphamezathine are given for comparison, these have not been previously recorded. With both drugs, the concentrations in tissues approximated to those in blood in magnitude and persistence. Figures showing tissue distribution in the nephrectomized cat are given in the following section.

TABLE II
TISSUE CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE
250 mg/kg orally in groups of 3 mice

Tissue	Concentrations in mg./100 g. of wet tissue after								
	30 min.	2 hrs	4 hrs	6½ hrs	19 hrs	31 hrs	48 hrs	72 hrs	96 hrs
Lung	14.5	16.9	19.6	17.6	16.3	10.1	2.6	3.8	2.1
Liver	15.1	13.6	12.9	15.0	14.6	4.0	2.7	2.5	1.1
Kidney	14.1	12.9	12.2	7.9	8.9	6.7	2.7	2.6	0.5
Spleen	7.0	6.8	9.5	9.5	10.8	5.5	1.6	0.5	0.6
Fat		9.8							

The urinary excretion of the drug in the mouse has been examined in two groups of three animals. Following the administration of 250 mg/kg orally, 46 and 32 per cent of the administered drug was excreted over three days in the two groups, and of these amounts 39 and 44 per cent respectively were acetylated. These results confirm the conclusion drawn from estimations of blood concentration after intraperitoneal injection, namely, that the drug is poorly absorbed in the mouse. The observation that, although much more persistent than sulphamezathine or sulphamerazine, sulphadimethoxypyrimidine gives lower blood concentrations than either of these compounds, can be explained on the same basis.

Experiments to determine the extent of conjugation of the new drug in the blood stream of the mouse are described below in the section dealing with acetylsulphadimethoxypyrimidine.

TABLE III
TISSUE CONCENTRATIONS OF SULPHAMEZATHINE
250 mg/kg orally in groups of 3 mice

Tissue	Concentrations in mg./100 g. of wet tissue after				
	30 min.	2 hrs	4 hrs	7 hrs	24 hrs
Lung	21.8	17.1	12.3	6.5	2.3
Liver	18.0	18.8	10.7	3.1	3.0
Kidney	19.9	20.8	17.3	13.2	1.6
Spleen	14.8	11.1	6.3	4.2	1.4
Fat	10.7	6.6	7.1	5.3	1.9

In species other than the mouse—The blood concentration time curves presented have in each case been chosen as typical from at least three available in the particular species. The drug was given orally as a solution of the sodium salt in the following amounts: dog, calf, sheep, 100 mg/kg.; rabbit, 150 mg/kg.; rat, chick, 200 mg/kg. The

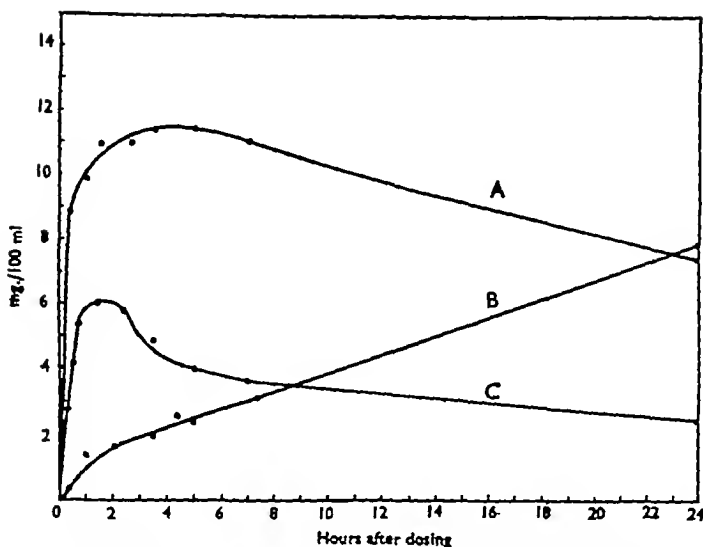


FIG 3—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 200 mg./kg. to the rat (A) and chick (B) and of 150 mg./kg. to the rabbit (C)

resultant blood concentrations are given in Figs 3 and 4. In the rabbit, the urinary excretion of the drug has been measured, and the amounts appearing as free amine and in conjugated form estimated (Table IV). Rabbit A, which received 150 mg./kg. of the drug, excreted approximately 80 per cent in the urine, of which some 47 per cent was conjugated.

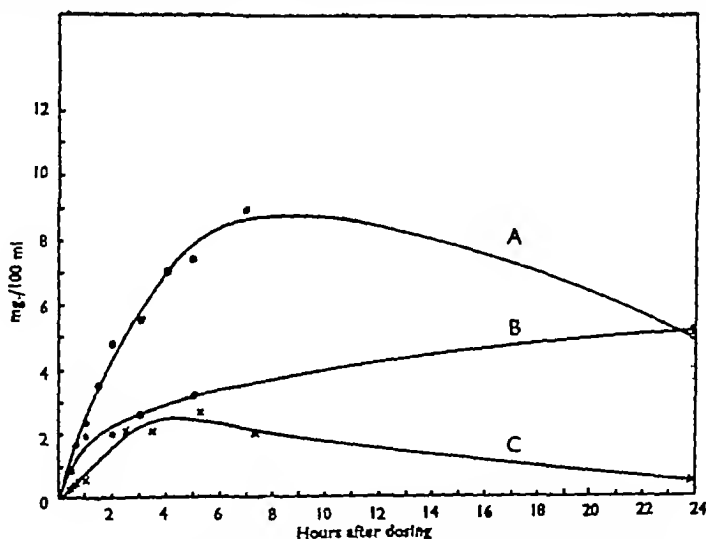


FIG 4—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 100 mg./kg. to sheep (A), calf (B) and dog (C)

TABLE IV

EXCRETION OF FREE AND CONJUGATED SULPHADIMETHOXYPYRIMIDINE IN THE URINE OF TWO RABBITS

Rabbit A (1.2 kg., dose 180 mg.)					Rabbit B (2.17 kg., dose 217 mg)				
Day	Free drug		Total drug		Day	Free drug		Total drug	
	mg	% of dose	mg	% of dose		mg.	% of dose	mg.	% of dose
1	62.7	34.8	120.3	66.8	1	35.5	16.3	134.9	62.1
2	5.7	3.2	13.8	7.7	2	5.4	2.5	14.8	6.8
3	5.8	3.2	6.4	3.6	3	7.6	3.5	13.7	6.3
4 + 5	3.1	1.7	5.9	3.3	4 + 5	2.2	1.0	4.3	2.0
Total	77.3	42.9	146.4	81.4	Total	50.7	23.3	167.7	77.2

The proportion of total drug excreted by rabbit B, which was given a smaller dose of 100 mg./kg., was similar, but the percentage conjugated was much higher (70 per cent). Despite the high recovery, indicative of efficient absorption from the gastro-intestinal tract, the maximum blood concentrations attained in the rabbit were appreciably lower than those reached in the rat and mouse although a similarly high degree of persistence was observed. The expression C_7 , obtained from Fig. 3, had a value of 14 hours for the rabbit, as against 17 hours for the rat and 16.5 hours for the mouse. In the sheep, calf, and chick the drug was slowly absorbed but persisted well, particularly in the last two. Absorption was poor in the dog and removal from the blood stream rapid ($C_7=2$ hours). Conjugation in rabbit and sheep is described in the section dealing with acetylsulphadimethoxypyrimidine.

The tissue distribution of the drug in nephrectomized cats has been estimated using the technique of Fisher, Troast, Waterhouse, and Shannon (1943). The results recorded in Table V are the means of three experiments and show the tissue/plasma ratio, they are compared with the figures obtained by Fisher *et al* for sulphamerazine and sulphadiazine.

TABLE V

DISTRIBUTION OF SULPHADIMETHOXYPYRIMIDINE IN THE BILATERALLY NEPHRECTOMIZED CAT

Drug	Tissue/plasma concentration ratios							Vol of distribution (as % of body weight)
	C.S.F	Brain	R.B.C.	Lung	Liver	Pancreas	Muscle	
Sulphadimethoxy-pyrimidine	0.23	0.23	0.12	0.40	0.68	0.35	0.24	39
Sulphamerazine*	0.38	0.35	0.45	0.56	0.76	0.47	0.39	45.8
Sulphamezathine*	0.31	0.21	0.53	0.60	0.63	0.44	0.45	82.5

*Data of Fisher *et al* (1943)

It will be seen that sulphadimethoxypyrimidine diffuses rather less into most tissues than the other sulphapyrimidines, and markedly less into the red blood cells, this may possibly be connected with the high protein binding of the drug (*vide infra*).

Antibacterial action in vitro

Comparisons of the antibacterial activities of sulphadimethoxypyrimidine, sulphamezathine and sulphadiazine were made by the method of Harper and Cawston

(1945) The medium used was Wright's broth containing 10 per cent (v/v) of lysed horse blood. Serial two-fold dilutions of the various sulphonamides in this medium were placed in 3 in \times $\frac{1}{4}$ in tubes in 0.5 ml amounts, and 0.5 ml of a 0.2×10^{-8} dilution in plain broth of a 24-hour culture of *Streptococcus pyogenes* Kruger strain, was added to each tube. (This inoculum gave a count of approximately 5×10^4 colonies per ml on blood agar.) The final

TABLE VI
COMPARATIVE ANTIBACTERIAL ACTIVITIES. RESULTS OF 48-HOUR PLATING ON BLOOD-AGAR
Organism *Streptococcus pyogenes*

Sulphonamide	Concentration—one part in				Control
	40,000	80,000	160,000	320,000	
Sulphadimethoxypyrimidine	—	—	+	+	+
Sulphamezathine	—	±	+	+	
Sulphadiazine	—	—	±	+	

medium thus contained 5 per cent of lysed horse blood and the concentration of the sulphonamides ranged from 1/40,000 to 1/320,000. All tubes were incubated at 37° C. for 48 hours. One loopful from each tube was removed and plated upon blood-agar. The end points were quite sharp and were recorded as — (no growth), ± (partial growth), and + (growth equal to control). The results are shown in Table VI. It will be seen that sulphadimethoxypyrimidine was intermediate in activity between the other two compounds.

Acute toxicity

When a suspension of sulphadimethoxypyrimidine was given orally to a group of 6 mice at the rate of 10 g./kg none died. The intravenous injection of solutions of the sodium salts of sulphamezathine and of sulphadimethoxypyrimidine gave the results shown in Table VII, which is a summary of two experiments. All doses were contained in a volume of 0.2 ml, and each injection took 1 min. to complete.

TABLE VII
THE TOXICITY OF SULPHAMEZATHINE AND SULPHADIMETHOXYPYRIMIDINE BY INTRAVENOUS
INJECTION OF THEIR SODIUM SALTS
Sixteen mice in each group. All mice observed for 5 days

Dose	Sulphamezathine	Sulphadimethoxy- pyrimidine
10 g./kg	16 died	16 died
0.9 " "	16 "	13 "
0.8 " "	13 "	12 "
0.7 " "	2 "	10 "
0.6 " "	2 "	4 "
0.5 " "	0 "	0 "

Chronic toxicity

Two separate experiments were carried out in which sulphadimethoxypyrimidine was administered as an aqueous dispersion once a day to rats for 28 days, at the rate of 1 g. per kg. body weight per day. The total number of rats in the two experiments was 54.

At the end of the period of administration of the drug the average final body weight of the treated animals was 130 per cent of their initial weight (from 110 g to 143 g), whilst the corresponding figure for equal-sized groups of matched control rats was 150 per cent.

Thirteen of the treated animals died during the period of treatment, or were killed because they were losing weight and were obviously ill, usually about 14 days from the start of the experiment. In several of these rats death was undoubtedly hastened by septic broncho-pneumonia brought on by maladministration of the doses of drug, but there were other deaths among rats whose lungs appeared normal. In the majority of the latter death could be ascribed to severe central necrosis of the liver lobules, sometimes affecting the whole lobule. It should be noted that the livers of 36 of the rats showed no abnormalities. Anaemia was very marked in many of the survivors, 5 of them giving readings of 29-46 per cent haemoglobin by the Sahli method. The spleens of many of the animals showed evidence of increased destruction of red blood corpuscles.

Although damage to the kidneys had been expected on account of the low solubility of this compound, such damage was seen in only 7 of the 54 animals examined. In only one of these was it severe, consisting of massive destruction of many convoluted tubules, amounting often to complete disintegration of the cell, with loss of nuclear staining and desquamation of the cells into the lumen of the tubule.

Therapeutic activity

Therapeutic experiments were carried out in mice infected with either *Streptococcus pyogenes* Kruger strain (Group A), *Streptococcus pneumoniae* Type I, or *Staphylococcus aureus*. The first two organisms were given intraperitoneally and the third intravenously. All drugs were administered by mouth as aqueous solutions or dispersions, the desired dose

TABLE VIII

THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH *Streptococcus pyogenes*, *Streptococcus pneumoniae* OR *Staphylococcus aureus*
Oral doses of 100 mg/kg twice daily for 3 days

Drug	<i>Streptococcus pyogenes</i>		<i>Streptococcus pneumoniae</i>		<i>Staphylococcus aureus</i>	
	No of deaths in 7 days	Mean survival time in days (max 7)	No of deaths in 7 days	Mean survival time in days (max 7)	No of deaths in 14 days	Mean survival time in days (max 14)
None (controls)	12	0.8	12	0.8	12	1.6
Sulphanilamide	12	1.0	12	1.2	10	5.0
Sulphapyridine	11	1.7	12	2.8	9	6.1
Sulphathiazole	12	1.0	12	2.1	8	7.2
Sulphamezathine	12	2.3	10	3.6	5	9.4
Sulphadiazine	11	3.8	12	4.3	4	11.2
Sulphamerazine	12	4.0	—	—	—	—
Sulphadimethoxy-pyrimidine	11	4.3	8	5.6	5	10.7

Streptococcus pyogenes Infecting dose 0.2 ml of a 1:10³ dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth, given intraperitoneally

Streptococcus pneumoniae Infecting dose 0.2 ml of a 1:10³ dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth, given intraperitoneally

Staphylococcus aureus Infecting dose 0.2 ml of a 1:2 dilution of a plain broth culture 18 hours old, given intravenously

being contained in a volume of 0.5 ml. The drugs were administered shortly before the infection and further doses were given 7, 24, 31, 48, 55, 72, and 79 hours after the infection. The dose chosen for all the drugs in the first experiment (100 mg./kg) was selected because experience had shown that, with streptococcal and pneumococcal infections, it permitted the various compounds to be ranged in order of activity on the basis of mean survival times. Table VIII summarizes the results of these experiments.

Taking all the experiments together, it will be seen that the increasing order of effectiveness of these compounds is approximately that in which they are arranged in the table.

In order to simulate more closely the conditions under which these drugs are used in clinical practice, another experiment was carried out in which infection of the mice preceded drug treatment by seven hours, in this experiment the mice were infected intraperitoneally with streptococci. The results are shown in Table IX.

The results of this experiment range the drugs in substantially the same order as before. In both experiments sulphadimethoxypyrimidine compares very favourably with any of the other drugs.

TABLE IX
THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH *Streptococcus pyogenes*
Infected at 10 a.m. and treated at 5 p.m. of the same day with 500 mg. drug/kg. No further doses given

Drug	No. of deaths in group	Mean survival time in days (maximum 7)
None (controls)	12	0.8
Sulphanilamide	12	0.8
Sulphapyridine	12	1.5
Sulphathiazole	12	1.0
Sulphamezathine	12	1.5
Sulphadiazine	12	2.3
Sulphamerazine	12	2.5
Sulphadimethoxypyrimidine	12	2.8

The infecting dose was 0.2 ml. of a 1×10^8 dilution of a culture of *Streptococcus pyogenes* prepared as in Table VIII.

Acetylsulphadimethoxypyrimidine

Estimation and occurrence—As indicated above, sulphadimethoxypyrimidine appeared in a conjugated form in the urine of experimental animals. Since hydrolysis gave a diazotizable amine, the conjugated product was assumed to be the acetyl derivative and all estimations were made against this compound as standard. The method of Rose and Bevan (1944) proved satisfactory for the estimation of acetylsulphadimethoxypyrimidine in urine, but it yielded low recoveries when known amounts of the compound were added to blood and tissue homogenisates. Variable recoveries of the order of 60 per cent were obtained from human blood and 30 per cent or less from mouse tissues. The recovery from blood was increased to 70 per cent by hydrolysis of the conjugated drug in whole blood before the precipitation of proteins. This could not be regarded as satisfactory, and further work

showed that adequate recoveries were obtained from blood and tissue by diluting to 1:150 before precipitating protein. A suitable aliquot was then taken, hydrolysed with dilute hydrochloric acid, diazotized and coupled with *N*- β -sulphatoethyl-*m*-toluidine. A coupling time of 30 min. was required, this is greater than that of the parent compound and probably indicates breakdown of the pyrimidine ring (cf. Frisk, 1943).

Estimations of free and total drug in the blood of mice receiving 250 mg. of sulphadimethoxypyrimidine/kg. orally, indicated the presence of traces only of conjugated amine. In the rabbit, on the other hand, high concentrations of conjugated drug were reached (Fig. 5). Similar results were obtained in the sheep, it being clear in both species that the acetyl derivative is similar in persistence to the free drug.

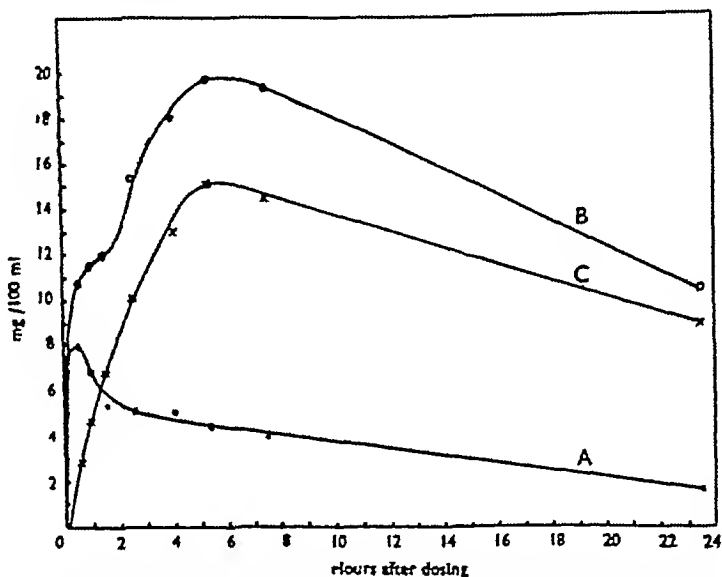


FIG. 5—Blood concentrations of free (A), total (B), and conjugated (C, by difference) sulphadimethoxypyrimidine in the rabbit following the oral administration of 250 mg./kg. orally

The administration of acetylsulphadimethoxypyrimidine was examined in the mouse. Four groups of three mice received 250 mg./kg. orally as a 1 per cent solution of the sodium salt. The mean blood concentration-time curves of free and total drug are shown in Fig. 6. That the observed hydrolysis of the acetyl derivative proceeds systemically and not in the lumen of the gut was readily shown by administering it intraperitoneally, when curves very similar to those of Fig. 6 were obtained. This facile hydrolysis of acetylsulphadimethoxypyrimidine undoubtedly accounts for its high activity, observed in the therapeutic experiments described below. In experiments with acetylsulphanilamide we found only traces of free drug in the blood following its administration in doses of 250 mg./kg., a result in agreement with its inactivity in therapeutic tests.

Therapeutic activity—Only activity against *Streptococcus pyogenes* in mice has been examined in this instance, the infecting inoculum being similar to that used in the experiments recorded in Table VIII. The dosage regime was slightly altered in that amounts of 250 mg./kg. mouse (as against 100 mg./kg. mouse of the free amine) were given twice daily for three days, beginning shortly before infection. Acetyl derivatives of other sulphonamide drugs were included for comparison. Two series of experiments were made in one

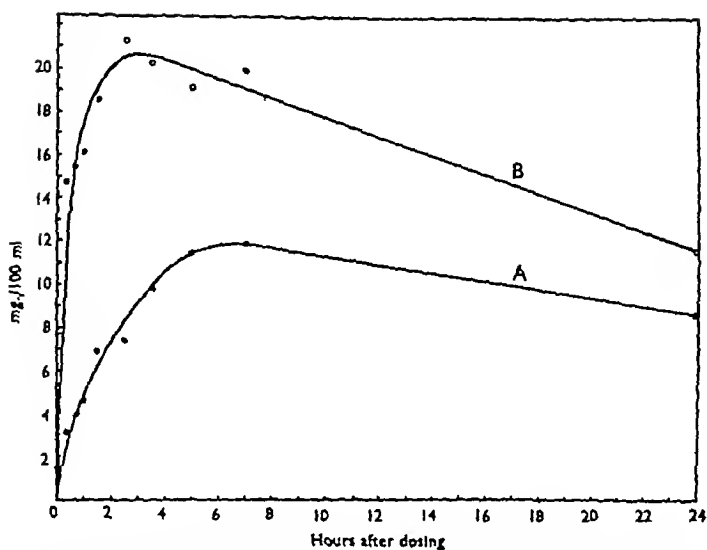


FIG 6—Blood concentrations of free (A) and total (B) sulphadimethoxypyrimidine following the oral administration in mice of 250 mg. of the acetyl derivative/kg

the drugs were administered by mouth, and in the other intraperitoneally. The average survival times are indicated in Table X. Groups of 6 mice only were employed in each experiment.

Since the order of relative effectiveness of the acetyl derivatives is the same by both routes of administration, it appears that in all cases they are largely absorbed as such, and

TABLE X

THERAPEUTIC RESULTS OBTAINED IN GROUPS OF 6 MICE INFECTED WITH *Streptococcus pyogenes* 250 mg/kg. twice daily for 3 days

Drug	Oral		Intraperitoneal	
	No of deaths in 7 days	Mean survival time (max. 7)	No of deaths in 7 days	Mean survival time (max. 7)
None (controls)	6	0.8	6	0.8
Acetylsulphanilamide	6	0.9	6	0.8
Acetylsulphapyridine	6	0.9	6	0.8
Acetylsulphadiazine	6	1.1	6	1.0
Acetylsulphathiazole	4	3.2	6	2.3
Acetylsulphamezathine	6	1.5	6	1.0
Acetylsulphamerazine	6	0.8	—	—
Acetylsulphadimethoxypyrimidine	6	5.0	4	5.0

The infecting dose was 0.2 ml. of a 10^6 dilution of a broth culture of *Streptococcus pyogenes* prepared as in Table VIII.

absorption is not preceded by extensive hydrolysis to the free amines in the gastro-intestinal tract. This accords with the view expressed above in the case of acetylsulphadimethoxypyrimidine following direct estimation of blood concentrations of the free amine

Physico-chemical properties

Solubility—The solubility determinations were made in water at 37° C. by the method of Rose, Martin, and Bevan (1943), increasing pH by the addition of sodium hydroxide solution. The concentration of drug in a sample withdrawn through a filter plug into a pipette was determined colorimetrically. With the acetyl derivative, de-acetylation was necessary before the colorimetric estimation, which depended upon a diazo reaction, could be made. The solubility curves so obtained are shown in Fig 7. In the pH range 6.0–7.0, the solubility of the free amine closely resembles that of sulphadiazine (Rose, Martin, and Bevan, 1943), thereafter the curve rises more steeply. Acetyl-sulphadimethoxypyrimidine differs from acetylsulphadiazine, however, in that it is less soluble than the parent amine in the pH range 6.0–7.3, but above the latter limit the acetyl derivative exhibits the greater solubility.

The acid dissociation constant—The acid dissociation constant has been measured by potentiometric titration of the saturated aqueous solution and

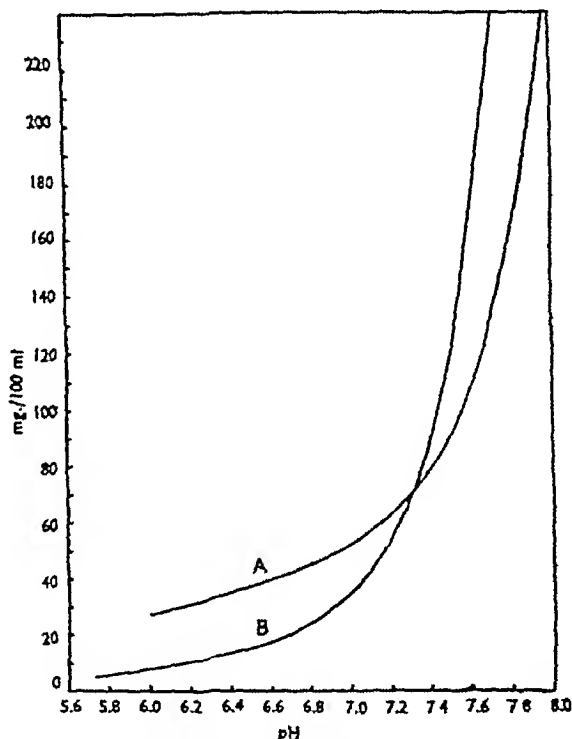


FIG 7—Solubility in water of sulphadimethoxypyrimidine (A) and of the acetyl derivative (B)

TABLE XI

PROTEIN BINDING OF SULPHADIMETHOXYPYRIMIDINE

Plasma or serum	Percentage of drug bound to protein	
	Ultrafiltration	Dialysis
Rat plasma	85	82
Rat serum	87	80
Human plasma	—	80
Mouse plasma	—	78
Cat plasma	79	—
Sheep serum	63	—
Rabbit serum	71.5	—
Horse serum	63	—

the pK_a value found to be 7.00. A solvent partition method gave the value 7.02. The pK_a values for other sulphanilamide derivatives of pyrimidine are known—e.g., sulphadiazine 6.48, sulphamerazine 7.06, sulphamezathine 7.37 (Bell and Roblin, 1942).

Protein binding—The association of the drug with blood proteins has been estimated by the equilibrium dialysis method of Davis (1943) and by ultrafiltration through collodion. Table XI shows the results obtained with various species.

It appears that sulphadimethoxypyrimidine is among the more highly bound sulphonamides, though a precise comparison is not possible, owing to the wide variation in values quoted for other drugs in the literature. The results are of the same order as the figure of 78 per cent quoted by van Dyke *et al* (1945) for the same compound.

SUMMARY AND CONCLUSIONS

1 The properties of 2-*p*-aminobenzenesulphonamido-4,6-dimethoxypyrimidine (sulphadimethoxypyrimidine) are described, and include water solubility data for the free amine and the acetyl derivative over a range of pH.

2 The drug is relatively non-toxic in mice and rats, rather more slowly absorbed than sulphadiazine, sulphamerazine, or sulphamezathine when given orally to mice, but markedly more persistent in the blood-stream than these three drugs. Absorption data are given for other experimental animals.

3 A high percentage of the absorbed drug is excreted by the mouse in conjugated form, but feeding the acetyl derivative (or injecting intraperitoneally) gives rise, after a few hours, to a concentration of the free amine in the blood almost equal to that attained by initial administration in the latter form.

4 The antibacterial activity *in vitro* of the drug against *Streptococcus pyogenes* is intermediate between that of sulphamezathine and sulphadiazine, but therapeutic activity against this organism in the mouse is, in general, at least equal to or slightly better than that shown by any of the other sulphapyrimidine derivatives, in conformity with the high persistence of the drug.

5 Acetylsulphadimethoxypyrimidine given orally or intraperitoneally to infected mice exerts an appreciable therapeutic effect, greater than that shown by the acetyl derivatives of the several other heterocyclic sulphonamides examined.

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2-*p*-AMINO BENZENESULPHONAMIDO-4 · 6-DIMETHOXYPYRIMIDINE ABSORPTION AND EXCRETION IN MAN

BY

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WITH NOTES ON A CLINICAL TRIAL IN PNEUMONIA

BY

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(Received January 1 1947)

The work of Gage, Martin, Rose, Spinks, and Tuey (1947) showed that a new sulphonamide, 2-*p*-aminobenzenesulphonamido-4 · 6-dimethoxypyrimidine or sulphadimethoxypyrimidine, had an unusual persistence when administered orally to animals. This fact, in conjunction with the observation that the antibacterial action *in vitro* and *in vivo* was of the same order as that of sulphadiazine, suggested that the compound merited a clinical trial, which was carried out in the medical wards of Crumpsall Hospital, Manchester. It was hoped that a sulphonamide which persisted in the blood for longer periods than other sulphonamides might be effective in the treatment of pneumonia in a single dose, or at most in doses once or twice daily.

METHODS

Sulphadimethoxypyrimidine was first administered to essentially normal patients, mostly convalescing from surgical and skin conditions, in doses ranging from 0.5 to 5 g. These patients were, at the time of testing, in fairly good general health and had no febrile condition, except in one patient (receiving 0.5 g) renal function was normal. Blood concentrations and recoveries in the urine are illustrated in the Table and Fig. 1. Two patients only were used for each dose level. The drug was also administered to a series of 80 patients, mostly suffering from pneumonia. One or two of these patients had other conditions normally requiring sulphonamide therapy. The results for 30 of these patients are shown in Figs. 2, 3, and 4, those obtained from the rest of the patients were similar although several patients had not received the full course before they were transferred to other treatments, penicillin or sulphamezathine, on clinical grounds.

Blood and urine sulphonamide estimations were made by the method of Rose and Bevan (1944). Twenty-four-hour collections of urine were made without preservative. For the blood urea, protein, phosphatase, and bilirubin estimations the methods of King (1946) slightly modified, were used.

RESULTS

Normal subjects showed a steady increase in maximum blood concentration and persistence with increasing dosage (Table, Fig 1) The maximum was

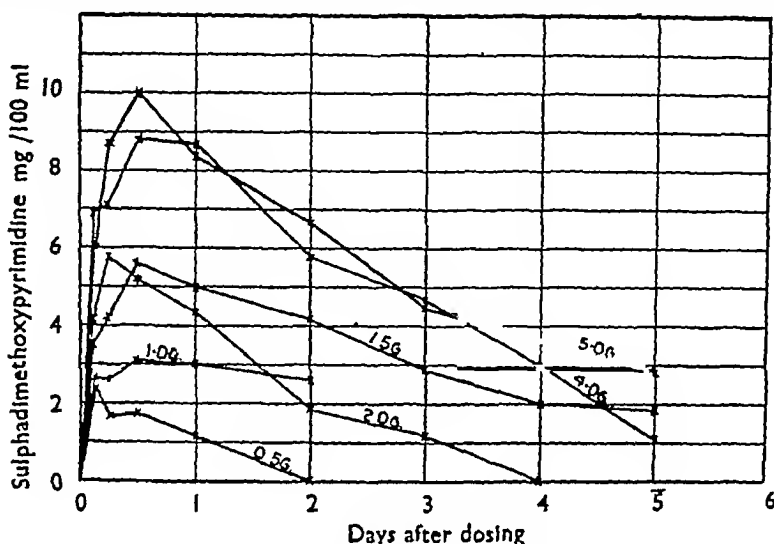


FIG 1—Blood concentrations of sulphadimethoxypyrimidine following the administration of single doses to normal subjects

TABLE

NORMAL PATIENTS MEAN BLOOD CONCENTRATIONS AND URINE RECOVERIES WITH SINGLE DOSES OF SULPHADIMETHOXYPYRIMIDINE

All readings are means for two patients (5.0 g dose, one patient only)

Dose g	Mean blood concentrations of free and, in parentheses, conjugated drug in mg./100 ml										Total urine recoveries of free and, in parentheses, conjugated drug	
	1 hr	2 hrs	3 hrs	6 hrs	12 hrs	24 hrs	2 days	3 days	4 days	5 days	mg	Per cent of dose
0.5	1.25 (0.1)	2.05 (0.05)	2.45 (0.2)	1.7 (0.05)	1.75 (1.25)	1.2 (0.55)	Trace (0.85)				74 (18)	14.8 (3.6)
1.0	1.4 (0.1)	2.15 (0.15)	2.65 (0.05)	2.65 (0.25)	3.1 (0.2)	3.0 (0)	2.6 (0.4)	Trace (1.6)			218 (46)	21.8 (5.3)
1.5	0.4 (0)	1.85 (1.05)	3.5 (1.0)	4.2 (0.95)	5.65 (0.7)	5.05 (0.85)	4.2 (0.7)	2.9 (0.2)	2.05 (0.35)	1.9 (0)	500 (77)	33.3 (4.4)
2.0	1.85 (0)	3.15 (0)	4.2 (0)	5.85 (0.5)	5.2 (0.6)	4.4 (0.35)	1.9 (0.2)	1.2 (0.25)	0 (Trace)		637 (252)	31.8 (12.6)
4.0	1.15 (0.25)	4.05 (0.1)	6.0 (0.25)	8.75 (0.65)	10.05 (0.45)	8.45 (0.6)	6.7 (0.4)	4.55 (0.45)	3.65 (0.25)	2.85 (0.2)	835 (241)	30.6 (6.0)
5.0	3.3 (0)	5.0 (0)	6.9 (0.3)	7.1 (0.1)	8.8 (0.2)	8.7 (0.3)	5.8 (0)	4.7 (0)	3.0 (0)	1.1 (0.5)	1202 (415)	24.0 (8.3)

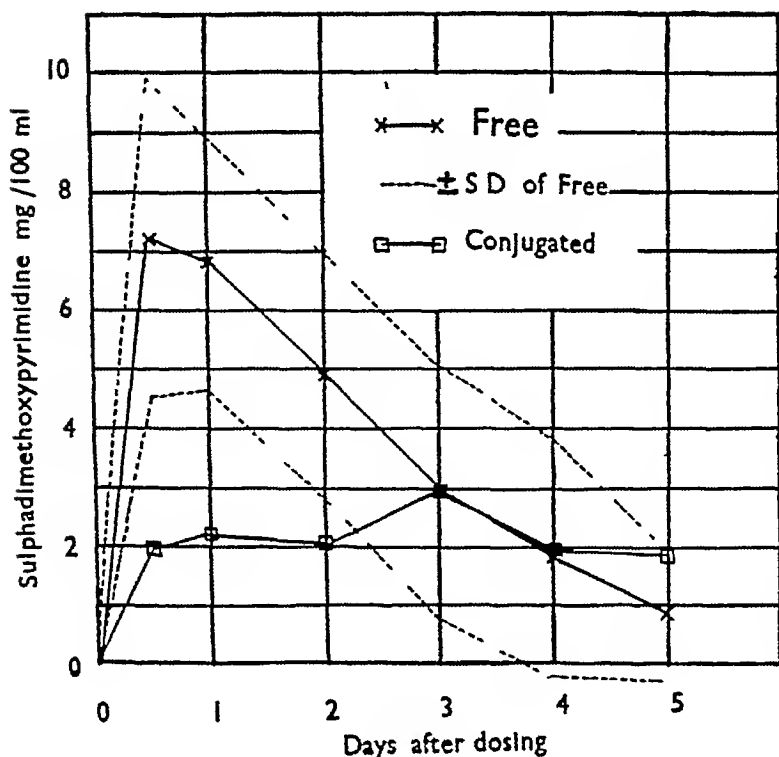


FIG 2—Blood concentrations of sulphadimethoxypyrimidine following the administration of a single dose of 5 g to pneumonia patients.

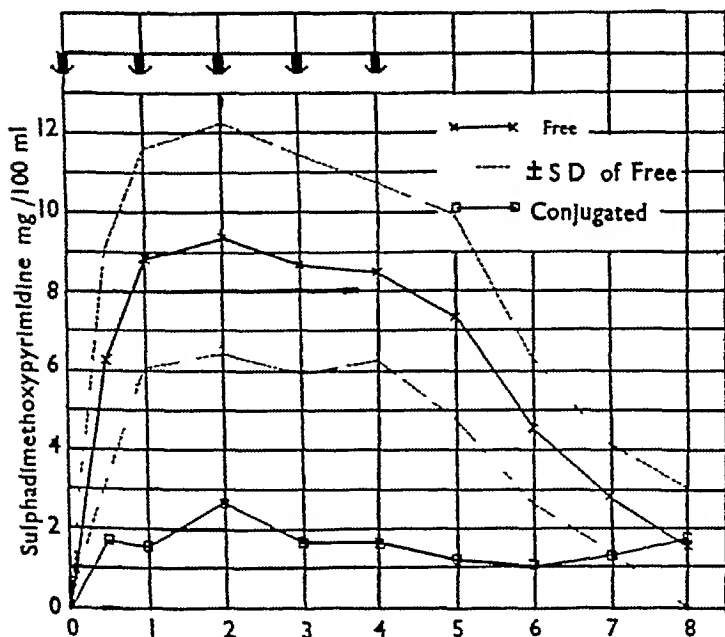


FIG 3—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g., and then 3 g., daily to pneumonia patients

attained at three hours after a dose of 0.5 g, but with higher dosage the maximum was at 12 hours. After a single dose of 4 g a blood concentration of 10.05 mg/100 ml was attained after 12 hours and 2.85 mg/100 ml persisted five days after the dose, a similar dose of sulphamezathine gave a maximum of 13.2 mg/100 ml, falling to traces within 24 hours. For patients suffering from pneumonia a single dose of sulphadimethoxypyrimidine gave on the average lower maxima and less well sustained blood concentrations (Fig 2). The average maximum attained after 5 g was 8.86 mg/100 ml, and only traces were detected in the blood after four days, although 2 mg/100 ml were still present after three days.

In patients treated with 5 g followed by 3 g every 24 hours, blood concentrations were usually well maintained (Fig 3). In a few cases, however, the drug concentration did not reach a level of 8 mg/100 ml, a concentration usually regarded as effective chemotherapeutically. Patients with a low concentration of free drug usually showed a high percentage of acetylation, a frequent finding with other sulphonamides.

Since clinical results were not completely satisfactory it was decided to try a dose of 5 g followed by 3 g every 12 hours (Fig 4). This dosage gave

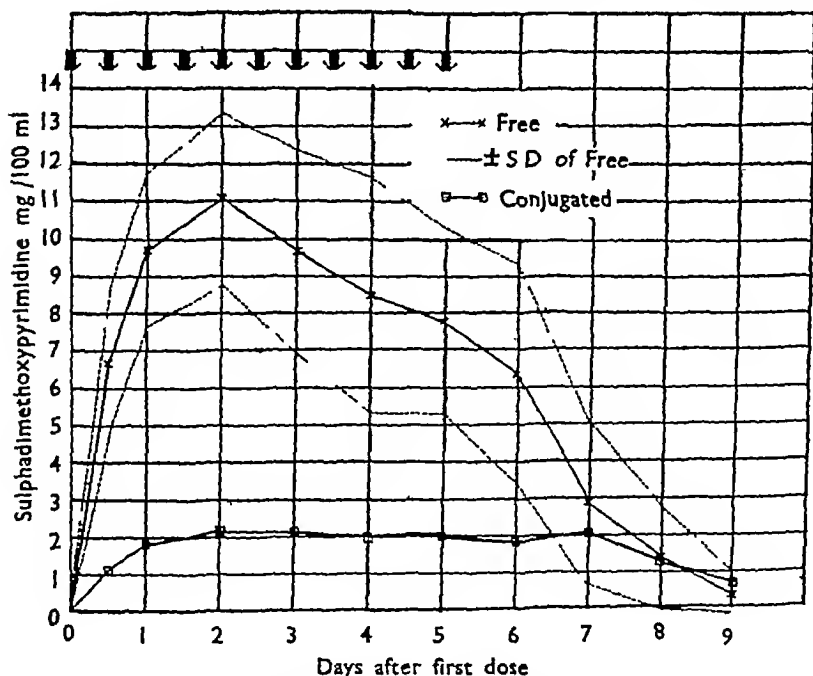


FIG 4—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g, and then 3 g, at twelve-hourly intervals, to pneumonia patients

slightly higher blood concentrations. All patients (except one who had only three doses) attained blood concentrations of over 8 mg/100 ml. In nearly all pneumonia patients receiving the drug, whether as single or repeated doses, free sulphadimethoxypyrimidine was still present in appreciable quantities three days after the last dose. In one patient with staphylococcal pneumonia and considerable renal damage (blood urea 100 to 150 mg/100 ml), sulphonamide was still present in measurable quantities 16 days after the last dose, though the blood urea had fallen slightly.

Samples of cerebrospinal fluid were obtained from two patients and contained sulphadimethoxypyrimidine concentrations, in one case of 58 per cent, and in the other of 31 per cent, of the simultaneous blood concentration.

The blood concentration of sulphadimethoxypyrimidine reached a slightly lower maximum than did sulphamezathine after similar doses, but this maximum was attained later and high blood concentrations persisted for a very much longer period. There was a tendency for the concentrations reached in the controls to be higher than those attained with similar doses of the drug in febrile patients. Persistence of the blood concentrations after the last dose was definitely less in the febrile patients.

The recovery of the drug in the urine was comparatively low. The average recovery was 32.9 per cent, 26.6 per cent being free and 6.6 per cent conjugated in the normal patients, as compared with 86.2 per cent for sulphamezathine (Clark *et al.*, 1943), 68 per cent for sulphadiazine (Reimhold *et al.*, 1941) and 57.3 per cent for sulphapyridine (Long and Feinstein, 1938). The percentage of the drug recovered as acetyl compound, both from blood and urine, was low. These results must in part be interpreted in the light of the results of Gage *et al.* (1947) who record only partial recovery of the drug after acid hydrolysis.

Crystals of the drug were seen in many of the urines passed, but no renal symptoms were observed directly referable to the presence of the crystals. In two patients with severe renal damage this damage could not be definitely assigned to the effect of sulphadimethoxypyrimidine. The crystals took the form of St. Andrew's crosses and were shown chemically to be free sulphadimethoxypyrimidine. When compared with the needle-shaped crystals often observed in the urine of patients receiving other sulphonamides, sulphadimethoxypyrimidine would not be expected to cause such extensive mechanical injury to the renal structures. Confirmatory evidence was provided by estimation of blood urea in 34 patients. The blood urea was usually about 40 mg/100 ml. on admission and in most cases tended to fall as the pneumonic condition improved. In no case was there a significant rise except in one woman dying within 48 hours of admission who was shown at autopsy to have severe chronic nephritis.

In chronic toxicity tests by Gage *et al.* (1947) large doses of sulphadimethoxypyrimidine in rats gave rise to severe central necrosis of the liver lobules, and it

was thought advisable to perform some liver function tests. Estimations of serum bilirubin, protein, and phosphatase were therefore made on nine patients. There was no change in the concentrations of these substances such as would indicate liver damage. It is realized that this series of tests would only show gross liver damage, and that minor impairment of function might pass unnoticed.

SUMMARY

- 1 Sulphadimethoxypyrimidine in man persists in the blood stream longer than do the other common sulphonamides after similar doses.
- 2 Acetylation of the drug is slight.
- 3 Single daily doses of sulphadimethoxypyrimidine will give blood concentrations of an order considered adequate for therapy in pneumonia.

CLINICAL TRIAL

Sulphadimethoxypyrimidine was given to 41 patients with pneumococcal lobar pneumonia, whose ages varied between 16 and 78 years.

An initial dose of 3 to 5 g, followed at intervals of 24 hours by two doses of 2 g, was found to give a blood level of 5-13 mg per 100 ml which was maintained for 40 to 60 hours. Eighteen patients were treated in this way.

Nine patients were given an initial dose of 5 g, followed by 3 g at intervals of 24 hours for four doses, a blood concentration of 5 to 14 mg per 100 ml being maintained for about 120 hours.

In thirteen patients an initial dose of 5 g was followed at 12-hour intervals by two doses of 3 g and then by 2 g doses for several days, maintaining blood concentrations between 7-16 mg per 100 ml. for four to six days. This method proved the best for maintaining adequate blood levels.

The drug was well tolerated and showed quite definite therapeutic value, but was not so effective as other sulphonamides used in series of similar cases (Don *et al*, 1940, Macartney *et al*, 1942, Ramsay *et al*, 1945).

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AN ASSESSMENT OF THE VALUE OF SUGGESTED THERAPIES FOR LEUCOPENIA

BY

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The subcutaneous injection of methyl-bis(β -chloroethyl)amine (a nitrogen mustard) into rabbits consistently produces a leucopenia, and the degree of this leucopenia is readily varied by variation in the dosage of methyl-bis(β -chloroethyl)amine. This phenomenon has been used as a method of assessment of the value of three suggested therapies for leucopenia. The therapies tested were

(i) Twenty-five per cent *p*-chloroxylenol in methylacetamide (CXM), which Zondek and Bromberg (1943) claim produces a leucocytosis lasting about four days in normal human subjects. In patients with typhoid fever the white cell count was maintained at a normal level by intramuscular injections of CXM, the count falling within 36 hours of discontinuing the injection.

(ii) The leucocytosis promoting factor (LPF) of Menkin and Kadish (1943). The latter state that 100 mg of this preparation in saline or phosphate buffer at pH 7.4 when given to animals either by cardiac puncture or by subcutaneous injection causes leucocytosis.

(iii) 1 g/100 c.c. aqueous sodium succinate, 0.1 c.c. of which, when injected subcutaneously into normal subjects or phthisic patients, is said to raise the white cell count (Hammett, Vessler, and Browning, 1917).

METHOD OF ASSAY

The leucopenia was produced by the subcutaneous injection of methyl-bis(β -chloroethyl)amine hydrochloride solutions (1 mg/c.c.). Rabbits (circa 2 kg.) and occasionally goats (circa 30 kg.) were used as the test animals. The therapies under test were given at various intervals after the methyl-bis(β -chloroethyl)amine hydrochloride injections, the efficacy of the therapies being judged by daily white cell counts performed between 10 a.m. and noon and before feeding.

RESULTS

(i) CXM—This was given by intramuscular injection. Zondek and Bromberg's dosage for man (i.e., two doses of 10 c.c. the first day and 10 c.c. daily for the next three days) was given to goats. In normal goats only a slight rise in the white cell count was produced, while in methyl-bis(β -chloroethyl)amine hydrochloride-poisoned animals leucopenia was not prevented and the mortality was actually doubled (Table I).

A similar picture was obtained with rabbits. In normal rabbits a slight leucocytosis was produced with small doses, but raising the dosage caused all

TABLE I

EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF GOATS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu mm on days						
			Z-1	Z	1	2	3	4	5
8		20 c.c. day Z 10 c.c. days 2 and 3	7.0	7.0	9.6	10.4	10.1	8.7	7.7
4	1 mg./kg day Z	—	9.9	11.0	10.0	3.0*	3.7	3.7	2.4*
4	1 mg./kg day Z	20 c.c. day 1 10 c.c. days 3 and 4		11.0	5.4	7.8*	4.6	2.9*	—**

* — one animal dead

the rabbits to die. With methyl-bis(β -chloroethyl)amine-poisoned rabbits leucopenia was not prevented and the mortality was increased (Table II)

TABLE II

EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu mm on days								
			Z-1	Z	1	2	3	4	5	6	8
4	—	0.5 c.c./kg day Z	9.2	9.3	12.3	12.2	9.3*	6.3			
3	—	1 c.c./kg day 1									
		5.0 c.c./kg day Z	7.9	7.1	6.9*	6.2*	3.0*				
4	1 mg./kg day Z	1 c.c./kg day 1		8.4	6.7	6.2*	1.2	2.4*	2.9	7.4	
5	1 mg./kg day Z	0.5 c.c./kg day 2		7.4	8.1	7.0	***				
		5 c.c./kg day 1					**				
5	1 mg./kg day Z	2 c.c./kg day 1	7.4	7.5	7.4	6.8	6.5	4.4**	3.1	3.6**	
		1 c.c./kg day 2									
5	1 mg./kg day Z	1 c.c./kg days 1 and 2	6.5	7.0	6.5	6.4	5.8	4.9	3.4	3.4*	3.8
10	1 mg./kg day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2

* — one animal dead.

(11) LPF—This was prepared from the inflammatory pleural exudates (produced by injection of turpentine) of rabbits and goats (LPF(R) and LPF(G)). When injected subcutaneously into normal rabbits or goats there was some evidence of the production of a slight leucocytosis, but with methyl-bis(β -chloro-

ethyl)amine hydrochloride-poisoned animals the leucopenia was not prevented and the death-rate was actually increased (Table III)

TABLE III
EFFECT OF LPF ON THE WHITE BLOOD CELL COUNTS OF GOATS AND RABBITS

Number of animals	Dosage nitrogen mustard	Therapy and dosage	Average white cell counts in thousands/cu mm on days								
			Z-1	Z	1	2	3	4	5	6	7
(a) RABBITS											
5	—	100 mg LPF(R) day Z and 10 mg day 1	10.5	11.0	11.0	9.6	10.0	10.4			
4	—	100 mg LPF(G) day Z	7.0	6.8	9.1	11.3	8.9	13.2	14.9	11.6	12.3
5	1 mg./kg day Z	100 mg LPF(R) day 1	9.6	9.7	9.6	4.8	4.6*	8.4**	8.2	10.9	10.0
3	1 mg./kg. day Z	100 mg LPF(R) day 1 and 2		8.1	8.7	5.9	1.2	1.3	5.0**	10.2	
5	1 mg./kg day Z	100 mg LPF(G) day 1	9.1	10.2	9.6	8.9	9.2**	9.8	13.0*	9.8	8.6
4	1 mg./kg day Z	100 mg LPF(G) day 1 and 2		7.5	7.0	3.9	1.0*	0.5	1.2	5.3	
10	1 mg./kg. day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2
(b) GOATS											
2	—	300 mg LPF(G) day Z and 1	7.3	5.6	12.9	11.5	10.8	9.0	9.8	8.4	
6	1 mg./kg day Z	300 mg LPF(G) day 1 and 2	8.1	7.9	14.9	9.9	7.3	5.5**	5.8**	5.4	
4	1 mg./kg. day Z	—	9.9	11.0	10.0	3.0*	3.7	3.7	2.4*	2.5	6.1

* — one animal dead.

(iii) *Sodium succinate*—Various quantities and concentrations of sodium succinate in aqueous solution were injected subcutaneously into normal rabbits

TABLE IV
EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF NORMAL RABBITS

Number of animals	Therapy dosage	Average white cell counts in thousands/cu mm on days								
		Z-1	Z	1	2	3	4	5	6	7
5	0.1 c.c. 1% solution day Z	8.6	8.9	10.9	11.9	12.0	9.3	9.2		
5	0.5 c.c. 1% solution day Z	9.2	9.6	11.9	13.1	12.1	9.5	9.2		
5	1 c.c. 1% solution day Z	9.8	8.1	11.1	11.9	12.0	13.1	9.2		
5	1 c.c. 1% day Z, twice daily, days 1, 2, 3	9.1	9.8	11.8	14.2	20.8	10.8	14.2	15.0	9.8
5	1 c.c. 10% solution day Z		15.5	14.3	12.4	12.2	13.4	8.9	11.9	13.6

and there was definite evidence of the production of a leucocytosis, a 1 g /100 c c. solution being better than a 10 g /100 c c concentration (Table IV) However, the sodium succinate solutions did not prevent the occurrence of leucopenia in methyl-bis(β -chloroethyl)amine hydrochloride-poisoned animals (Table V)

TABLE V
EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF
NITROGEN MUSTARD POISONED RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu mm on days								
			Z-1	Z	1	2	3	4	5	6	7
10	1 mg./kg. day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2
15	1 mg./kg day Z	1 c.c. 1% day 1	9.6	9.4	11.4	10.2	5.7	6.0*	10.2	10.5	
5	1 mg./kg. day Z	1 c.c. 1% at Z + 6 hours	—	12.0	13.9	10.2	6.9	6.6	12.7	—	—
10	1 mg./kg. day Z	0.5 c.c. 1% day 1	9.6	10.3	10.7	11.5	10.0*	11.3	11.8	12.0	10.6
5	1 mg./kg day Z	1 c.c. 10% day 1	8.7	9.0	4.5	4.2	5.7	3.3**	—	—	—
10	2 mg./kg day Z	—	10.5	14.0	12.5*	11.1*	9.2	3.9	11.7*	11.5	15.3
5	2 mg./kg day Z	1 c.c. 1% days 1, 2, 3 and 4	13.2	13.6	14.9	16.7	6.7	10.4	16.8	12.9	—
10	2 mg./kg day Z	1 c.c. 1% days 1-6	9.5	12.7	8.9*	6.9	5.1	2.7**	13.4*	11.9	7.9
5	2 mg./kg. day Z	5 c.c. 10% day 1	8.4	10.1	8.8	3.8	0.4	0.3*	3.7		
5	2 mg./kg. day Z	1 c.c. 1% at Z + 6 hours	9.2	8.4	14.5	4.2	0.9*	3.5**			

* — one animal dead

SUMMARY

When assayed on rabbits or goats poisoned with methyl-bis(β -chloroethyl)-amine hydrochloride (given by subcutaneous injection), the following substances were ineffective in preventing leucopenia

1 *p*-chloroxylenol in methylacetamide—this preparation, indeed, was found to be toxic in the recommended dosage

2 The leucocytosis-promoting factor of Menkin.

3 An aqueous solution of sodium succinate

I am indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish this paper

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SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DIISOPROPYL FLUOROPHOSPHONATE

BY

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The inhibition of cholinesterase by fluorophosphonates was discovered in 1941 by Adrian, Feldberg, and Kilby (1947), when they examined dimethyl fluorophosphonate. In 1941 McCombie and Saunders prepared diisopropyl fluorophosphonate and Adrian *et al* (1942), as well as Mackworth (1942) found that it had an even stronger inhibitory action on cholinesterase than the dimethyl ester. At that time it was not known that there were two enzymes, true cholinesterase and pseudo-cholinesterase (Mendel and Rudney, 1943a), which are not necessarily affected similarly by inhibitors (Mendel and Rudney, 1944, Hawkins and Gunter, 1946). In the experiments to be reported in this paper it will be shown that diisopropyl fluorophosphonate, unlike eserine or prostigmine (Hawkins and Mendel, 1946, and unpublished experiments), exhibits a much stronger inhibitory action on pseudo-cholinesterase than on true cholinesterase. With low concentrations of diisopropyl fluorophosphonate it is therefore possible to inhibit pseudo-cholinesterase selectively without affecting true cholinesterase.

The possibility of such selective inhibition of pseudo-cholinesterase by diisopropyl fluorophosphonate was suggested by the following two observations.

(1) Bodansky (1945) as well as Mazur and Bodansky (1946) found that on exposure of human beings to low concentrations of the vapour of diisopropyl fluorophosphonate almost complete inhibition of cholinesterase activity in the plasma could be obtained without causing serious distress. It should be borne in mind, however, that human plasma contains predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943) and that the inhibition of this enzyme, as shown by Hawkins and Gunter (1946), will not interfere with the destruction of acetylcholine released by nervous activity. These workers found that certain concentrations of a prostigmine analogue, the dimethylcarbamate of 2-hydroxy-5-phenyl-benzyltrimethylammonium bromide (Hoffman-LaRoche Nu-683), are capable of inhibiting completely the activity of pseudo-cholinesterase *in vitro* without affecting significantly that of true cholinesterase. This

compound, when injected into dogs in amounts sufficient to inhibit pseudo-cholinesterase almost completely, elicits no symptoms indicative of the accumulation of acetylcholine, such symptoms appear only if the dose injected is sufficiently large to depress the activity of the true cholinesterase as well. Pseudo-cholinesterase, therefore, plays no essential role in the hydrolysis of acetylcholine *in vivo*.

(2) Bodansky and Mazur (1946) and Mazur and Bodansky (1946) found that the concentration of diisopropyl fluorophosphonate necessary for the inhibition of cholinesterase varied according to the enzyme preparations used, the negative logarithm of the molar concentration of diisopropyl fluorophosphonate necessary to produce a 50 per cent inhibition of the activity towards acetylcholine ($1.5 \times 10^{-2}M$) was 7.7 and 8.1 for human and horse serum respectively, the corresponding value for rabbit serum was 4.1, and the values for red blood cells and brain varied between 5.2 and 6.0. Since the sera of man and the horse contain predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943), rabbit serum mainly true cholinesterase (Mendel and Rudney, 1945), and brain and red blood cells throughout the animal kingdom true cholinesterase only (Mendel and Rudney, 1943a, 1943b), the results obtained by Mazur and Bodansky can be interpreted as indicating that pseudo-cholinesterase is approximately 100 times more sensitive to the inhibitory action of diisopropyl fluorophosphonate than is true cholinesterase.

METHODS

Cholinesterase activity was measured manometrically by Warburg's method at 37°C in $2.5 \times 10^{-2}M$ $NaHCO_3$ saturated with 5 per cent CO_2 in N_2 . The diisopropyl fluorophosphonate was added to the bicarbonate medium containing the enzyme preparation in the main compartment of the Warburg flask, the substrate being placed in the side arm. After the enzyme preparation had been shaken for 15 min to attain temperature equilibrium the substrate was tipped into the main compartment. From a stock solution, freshly prepared every third day, of diisopropyl fluorophosphonate ($10^{-2}M$) in propylene glycol, greater dilutions were made with distilled water as required. The final concentration of propylene glycol present in the experimental vessels caused by itself no inhibition of the cholinesterases.

The activities of the true and pseudo-cholinesterases were measured as described by Mendel, Mundell, and Rudney (1943), by the rates of hydrolysis of acetyl- β -methylcholine and benzoylcholine respectively.

RESULTS

In vitro—Preliminary experiments were carried out to determine the inhibitory action of diisopropyl fluorophosphonate on enzyme preparations containing either only true cholinesterase or only pseudo cholinesterase. This was done in order to ascertain whether the substrate acetylcholine could be replaced by acetyl- β -methylcholine in measuring the activity of true cholinesterase, or by benzoylcholine in measuring the activity of pseudo cholinesterase, without affecting the percentage inhibition by diisopropyl fluorophosphonate. Haemolysed dog erythrocytes were used as a source of true cholinesterase, and an extract of dog pancreas as a source of pseudo-cholinesterase. As shown in Table I, the degree of

inhibition of true cholinesterase and pseudo-cholinesterase by diisopropyl fluorophosphonate is not altered when acetyl- β -methylcholine or benzoylcholine respectively are substituted for acetylcholine

TABLE I

INHIBITION OF PSEUDO-CHOLINESTERASE AND TRUE CHOLINESTERASE BY DIISOPROPYL FLUOROPHOSPHONATE, USING VARIOUS SUBSTRATES

Enzyme preparation	Substrate*	Molar concentration of diisopropyl fluorophosphonate	Percentage inhibition of enzymatic activity
True cholinesterase (haemolysed dog erythrocytes)	Ach. $1.2 \times 10^{-3}M$	1×10^{-7}	4
	Mch. $3 \times 10^{-3}M$	1×10^{-7}	3
	Ach. $1.2 \times 10^{-3}M$	5×10^{-7}	25
	Mch. $3 \times 10^{-3}M$	5×10^{-7}	26
Pseudo-cholinesterase (suspension of dog pancreas)	Ach. $6 \times 10^{-3}M$	1×10^{-8}	87
	Bch. $6 \times 10^{-3}M$	1×10^{-8}	86
	Ach. $6 \times 10^{-3}M$	5×10^{-8}	100
	Bch. $6 \times 10^{-3}M$	5×10^{-8}	100

* Ach. = acetylcholine, Mch. = acetyl- β -methylcholine, Bch. = benzoylcholine.

Moreover, the presence of pseudo-cholinesterase does not interfere with the inhibition of the true cholinesterase. Table II shows that the hydrolysis of acetyl- β -methylcholine by the true cholinesterase of haemolysed human erythrocytes is inhibited 26 per cent by $5 \times 10^{-8}M$ diisopropyl fluorophosphonate. When highly purified pseudo-cholinesterase prepared from horse serum is mixed with the haemolysed erythrocytes in an amount possessing an activity approximating that of the pseudo-cholinesterase of human plasma, no diminution of the inhibitory action of diisopropyl fluorophosphonate on true cholinesterase is observed.

In subsequent experiments, therefore, acetyl- β -methylcholine and benzoylcholine could be used to estimate separately the activities of the two cholinesterases in enzyme preparations which in most cases contained a mixture of both.

TABLE II

INHIBITORY ACTION OF DIISOPROPYL FLUOROPHOSPHONATE ON TRUE CHOLINESTERASE IN THE PRESENCE OF PSEUDO-CHOLINESTERASE

Type of cholinesterase	Molar concentration of diisopropyl fluorophosphonate	Activity* (μl CO ₂ /15 min.)	Percentage Inhibition
True cholinesterase (haemolysed human erythrocytes)	—	100.0	—
True cholinesterase (haemolysed human erythrocytes)	5×10^{-8}	74.0	26.0
True cholinesterase (haemolysed human erythrocytes) in the presence of pseudo-cholinesterase† (purified horse serum)	5×10^{-8}	74.5	25.5

* Substrate in all cases acetyl- β -methylcholine ($3 \times 10^{-3}M$)

† The pseudo-cholinesterase from horse serum was kindly supplied by Miss F. Strelitz, who purified it according to her method (Strelitz, 1944). This preparation exhibited no activity towards acetyl- β -methylcholine.

The enzyme preparations tested were the plasma of man, dog, cat, rat, rabbit, and sheep. Sheep plasma contains no pseudo-cholinesterase, while the plasma of the other species contains both cholinesterases, although in different proportions. For each enzyme preparation, with the exception of sheep plasma, the minimal concentration of diisopropyl fluorophosphonate required to cause complete inhibition of pseudo-cholinesterase activity was determined, using benzoylcholine as substrate. The inhibitory action of this concentration of diisopropyl fluorophosphonate on the true cholinesterase in the plasma was then examined, using acetyl- β methylcholine as substrate.

The results of these experiments are shown in Table III. Although pseudo-cholinesterase is inhibited completely in all instances, the true cholinesterase is inhibited only partially—35, 34, and 33 per cent in human, rabbit, and rat plasma respectively, and only 7 per cent in dog plasma.

TABLE III
SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DIISOPROPYL FLUOROPHOSPHONATE

Source of enzyme	Substrate*	Molar concentration of diisopropyl fluorophosphonate	Activity (expressed as μ l CO ₂ evolved by 1 ml plasma in 15 min.)		Percentage inhibition
			Without inhibitor	With inhibitor	
Human plasma	Ach $6 \times 10^{-3}M$	1×10^{-4}	1280	13	99
	Mch $3 \times 10^{-3}M$		26	17	35
	Bch $6 \times 10^{-3}M$		570	0	100
Dog plasma	Ach $6 \times 10^{-3}M$	5×10^{-4}	590	60	90
	Mch $3 \times 10^{-3}M$		82	76	7
	Bch $6 \times 10^{-3}M$		294	0	100
Cat plasma	Ach $6 \times 10^{-3}M$	5×10^{-4}	426	14	96
	Mch $3 \times 10^{-3}M$		30	25	16
	Bch $6 \times 10^{-3}M$		109	0	100
Rat plasma	Ach $6 \times 10^{-3}M$	1×10^{-4}	123	16	87
	Mch $3 \times 10^{-3}M$		54	36	33
	Bch $6 \times 10^{-3}M$		20	0	100
Rabbit plasma	Ach $6 \times 10^{-3}M$	5×10^{-7}	45	20	56
	Mch $3 \times 10^{-3}M$		47	31	34
	Bch $6 \times 10^{-3}M$		4	0	100
Sheep plasma	Ach $6 \times 10^{-3}M$	5×10^{-7}	13	10	23
	Mch $3 \times 10^{-3}M$		14	11	22
	Bch $6 \times 10^{-3}M$		0	0	—

* Ach. — acetylcholine
Mch — acetyl- β -methylcholine
Bch — benzoylcholine } all in the form of the chloride

Besides disclosing the difference between the sensitivities of pseudo-cholinesterase and true cholinesterase towards diisopropyl fluorophosphonate, these experiments show that when acetylcholine serves as substrate, the inhibition brought about by diisopropyl fluorophosphonate depends on the proportion of true cholinesterase and pseudo-cholinesterase present in the plasma, the greater the content of pseudo-cholinesterase, the greater the discrepancy between the inhibition of true cholinesterase and the inhibition observed when acetylcholine

is the substrate, conversely, the lower the pseudo-cholinesterase activity, the closer the parallelism between the inhibition of true cholinesterase and the inhibition of the acetylcholine hydrolysis

In vivo—Mazur and Bodansky found that in human beings exposed to diisopropyl fluorophosphonate, an inhibition of 98–99 per cent of the activity of the plasma towards acetylcholine did not result in symptoms of acetylcholine accumulation. In the present series, animals were injected intramuscularly with diisopropyl fluorophosphonate in order to ascertain whether in species, the plasma of which contains true cholinesterase and pseudo-cholinesterase in a proportion different from that in human plasma, there is also no correlation between the inhibition of the activity of the plasma towards acetylcholine and the symptoms to be expected from this inhibition.

Rabbits were chosen as experimental animals because the plasma of this species contains a smaller proportion of pseudo-cholinesterase to true cholinesterase, and therefore (see Table III) the discrepancy between the degree of inhibition of the activity towards acetylcholine, on the one hand, and towards acetyl- β -methylcholine, on the other, is not so pronounced as with human plasma, in which pseudo-cholinesterase predominates.

It will be seen from the typical experiment outlined in Table IV that rabbits receiving intramuscular injections of diisopropyl fluorophosphonate display their first symptoms of acetylcholine poisoning (i.e., masticatory movements of the jaws and slight generalized fibrillation) at a time when an appreciable activity (18 per cent) of the plasma towards acetylcholine is still present. These results confirm Mazur and Bodansky's findings in their experiments with rabbits. However, these authors did not attempt to explain why in rabbits symptoms of acetylcholine poisoning appear when the cholinesterase of their serum still displays a considerable activity towards acetylcholine, whereas in man an almost complete inhibition of the activity of the plasma towards acetylcholine causes no symptoms of serious distress.

TABLE IV

RELATIONSHIP BETWEEN THE INHIBITION OF CHOLINESTERASES BY DIISOPROPYL FLUOROPHOSPHONATE AND THE ONSET OF SYMPTOMS OF ACETYLCHOLINE POISONING

Rabbit II—2.5 kg

- 11.26 Activity of plasma tested.
- 11.27 0.65 mg. diisopropyl fluorophosphonate* in saline injected intramuscularly
- 11.29 localized twitching of hind leg at site of injection
- 11.40 masticatory movements, which continued until
- 11.49 0.13 mg. diisopropyl fluorophosphonate in saline intramuscularly
- 11.56 generalized fibrillation.
- 11.57 chewing, swallowing and fibrillation, activity of plasma tested.

Time	Activity (expressed as μ l CO ₂ evolved by 1 ml. plasma in 15 min) towards					
	Bch † ($6 \times 10^{-3}M$)	Inhibition %	Mch. † ($3 \times 10^{-3}M$)	Inhibition %	Ach. † ($6 \times 10^{-3}M$)	Inhibition %
11.26	61	—	62.8	—	69.5	—
11.57	0	100	17.3	73	13.3	82

* An initial dilution (1 in 500) was made with propylene glycol

† Bch. = benzoylcholine, Mch. = acetyl- β -methylcholine, Ach. = acetylcholine.

DISCUSSION

The plasma of most species contains, in varying proportions, a mixture of two enzymes pseudo-cholinesterase, which plays no essential role in the hydrolysis of acetylcholine *in vivo*, and true cholinesterase, the inhibition of which results in symptoms of acetylcholine poisoning. The experiments reported here have shown that appropriate concentrations of diisopropyl fluorophosphonate completely inhibit pseudo-cholinesterase without affecting the true cholinesterase significantly (see Table III).

Since acetylcholine is hydrolysed by both cholinesterases, measurements with acetylcholine as substrate can yield no information about the contribution made by each of these enzymes to the total activity, and the extent of inhibition of the activity towards acetylcholine in the presence of a selective inhibitor of pseudo-cholinesterase will depend on the relative proportions of pseudo- and true cholinesterases in the mixture which is being tested. Therefore, the degree of inhibition of acetylcholine hydrolysis by diisopropyl fluorophosphonate is no index of the inhibition of the true cholinesterase.

In the light of the above facts it is not surprising that human beings exposed to low concentrations of diisopropyl fluorophosphonate exhibit no symptoms indicative of acetylcholine accumulation when their plasma has lost 98–99 per cent of its original activity towards acetylcholine, 99 per cent of the activity of human plasma towards acetylcholine ($6 \times 10^{-2}M$) is due to pseudo-cholinesterase, true cholinesterase accounting only for about 1 per cent of the total activity (Mendel, Mundell, and Rudney, 1943). Consequently, when diisopropyl fluorophosphonate causes a 98–99 per cent inhibition of the activity of human plasma towards acetylcholine, the inhibition of the pseudo-cholinesterase activity should be complete, while the activity of the true cholinesterase may be depressed less than 35 per cent (see Table III).

The results are entirely different with animals whose plasma contains predominantly true cholinesterase (e.g., rabbits). The hydrolysis of acetylcholine by the plasma of such animals is due mainly to the true cholinesterase, therefore, when a 98 per cent inhibition of the activity of their plasma towards acetylcholine is achieved, it must be the true cholinesterase which is inhibited to a great extent. Consequently, symptoms of acetylcholine poisoning should set in at a much lower level of inhibition of acetylcholine hydrolysis than would be the case in species, such as man, where the hydrolysis of acetylcholine by the plasma is due mainly to pseudo-cholinesterase. Indeed, our experiments with rabbits have shown that the injection of diisopropyl fluorophosphonate leads to parasympathomimetic symptoms and fibrillation at a time when the activity of the plasma towards acetylcholine is inhibited not more than 80–82 per cent. In sheep, whose plasma contains true cholinesterase only, these symptoms would probably appear at a still lower level of inhibition of the acetylcholine hydrolysis. It would seem, therefore, that the higher the ratio of true cholinesterase to

pseudo-cholinesterase, the lower the degree of inhibition of acetylcholine hydrolysis prevailing at the time of onset of symptoms

As mentioned previously, it is the inhibition of true cholinesterase which results in the appearance of symptoms of acetylcholine poisoning. On the basis of experiments in which the level of true cholinesterase activity was correlated with the appearance of symptoms after the injection of eserine, Gunter and Mendel (1945) concluded that the body possesses a surplus of this enzyme, they observed no ill-effects until the activity of the true cholinesterase was inhibited 70-80 per cent. Similarly, Hawkins and Gunter (1946) found that symptoms of acetylcholine accumulation made their first appearance in dogs when the true cholinesterase activity of their plasma had been depressed to 23 per cent of its original level. Koelle and Gilman (1946) reported only slight parasympathomimetic symptoms in rats when the activity of the true cholinesterase in the brain had been depressed to 21-28 per cent of the normal by intramuscular injection of diisopropyl fluorophosphonate, and in the present study symptoms of acetylcholine accumulation appeared in rabbits when the activity of the true cholinesterase in the plasma had been depressed to 27 per cent of its original level (see Table IV).

Therefore, diisopropyl fluorophosphonate, in order to produce symptoms of acetylcholine poisoning, must be present in a concentration which is sufficient to remove the true cholinesterase in excess of that required for normal function. To estimate to what extent this objective has been achieved by injection of, or exposure to, diisopropyl fluorophosphonate it is necessary to determine the degree of inhibition of the activity towards acetyl- β -methylcholine. The use of acetylcholine as substrate would yield no such information except in the rare cases in which pseudo-cholinesterase is absent or is present in negligible amounts only.

SUMMARY

1 Although diisopropyl fluorophosphonate inhibits both true and pseudo-cholinesterases, higher concentrations are required for the inhibition of true cholinesterase than of pseudo-cholinesterase. With appropriate concentrations of diisopropyl fluorophosphonate it is therefore possible, in a mixture of both enzymes, to inhibit selectively the activity of pseudo-cholinesterase without affecting that of true cholinesterase.

2 Acetylcholine is hydrolysed *in vitro* not only by true cholinesterase, but also by pseudo-cholinesterase, therefore, measurements of cholinesterase activity in which acetylcholine is used as substrate cannot be used to correlate the degree of inhibition of true cholinesterase by diisopropyl fluorophosphonate and the effects resulting from this inhibition *in vivo*. Since true cholinesterase is the enzyme responsible for the hydrolysis of acetylcholine released at nerve endings, it is the degree of inhibition of true cholinesterase which must be determined.

when a correlation between anti-cholinesterase action and pharmacological effects is sought.

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A PRELIMINARY REPORT OF THE TOXICITY AND THE ASSOCIATED BLOOD CONCENTRATIONS OF PALUDRINE* IN LABORATORY ANIMALS

BY

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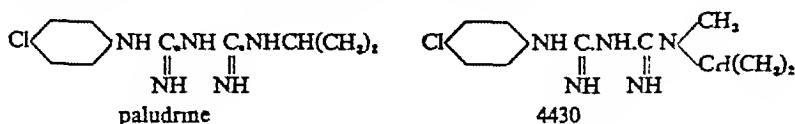
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During the preparatory work before paludrine (4888) was administered to human beings the usual investigations were made of its toxicity in various laboratory animals. Early in the work we recognized that different species of animals behaved differently towards it, the rat and the mouse, for example, appeared more susceptible than the chick. We also recognized that the differences were possibly not due to a difference in susceptibility to unchanged drug, but more likely to a difference in its metabolism and the liberation of greater or less amounts of toxic by-products. Sufficient work was done to justify giving the drug to human beings, and the Liverpool workers (Adams, Maegraith, King, Townshend, Davey, and Havard, 1945) pursued their investigations on the assumption that human beings might react like the mouse and the rat, which are amongst the most susceptible of the laboratory animals. It was for this reason that, in the beginning, paludrine was given only twice daily, and doses were progressively increased by amounts not greater than 25 mg. Quite quickly it was shown that man must be classed amongst the least susceptible animals.

The laboratory results have not been published earlier because it was hoped that a fuller investigation would be made. Unfortunately, this will take longer than was planned, and because paludrine has now been sent to many laboratories in different parts of the world we are making the preliminary results available without further delay for the convenience of other workers.

The constitution of paludrine (base) is given below



Two salts were used in the experiments, the monoacetate and the monohydrochloride. The former contains 81 per cent by weight of the base and is soluble

* Paludrine is the registered name for N₁-p-chlorophenyl-N₂-isopropylbiguanide

to the extent of about 2 per cent in water, the latter contains 87.4 per cent by weight of the base and is about half as soluble. Solutions of either salt are stable when boiled. The figures quoted in the text, unless it is stated to the contrary, refer to the salts. The monoacetate was used in the intravenous and intraperitoneal tests, the monohydrochloride in the oral tests.

Acute toxicity

This was measured in the usual way. Solutions were administered orally by means of a catheter tube, and intravenously or intraperitoneally by rapid (3 sec.) injection. The results are given in Table I. For most species of animal three sets of figures are quoted which give, respectively, approximately the largest dose permitting all animals to live (LD₀), the dose which kills approximately half the experimental animals (LD₅₀) and approximately the smallest dose which kills all (LD₁₀₀).

TABLE I
ACUTE TOXICITY OF PALUDRINE IN LABORATORY ANIMALS

Species	Route	LD ₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₁₀₀ (mg/kg)
Chick (wt 50 g)	Oral	200	400-600	100
	i.v.	40	60-80	
Mouse (wt 18-22 g)	Oral	50	60-80	100
	i.v.	10	20-30	40-50
	i.p.	10	20-30	40-50
Rat (wt 100 g)	Oral	80	100-150	60
	i.v.	20	40	
	i.p.	20	40	
Rabbit (wt 1.5 kg)	Oral	30	circa 150	
	i.v.		circa 50	

The intravenous or intraperitoneal injection of paludrine into both rats and mice is associated with delayed deaths, a point which is of much interest. It is best emphasized by comparing the results of an intravenous test using this drug with one using a closely related substance (4430), which differs only by a methyl group (see formula above). The results are given in Table II.

TABLE II
COMPARISON OF THE RESULTS FOLLOWING THE RAPID INTRAVENOUS INJECTION OF 4430 AND PALUDRINE INTO MICE

Dose	Results	
	4430	Paludrine
100 mg/kg 80 mg/kg.	6/6 mice dead within 3 min 5/6 mice dead within 3 min, survivor alive 5 days later	12/12 mice dead within 3 min 9/18 mice dead within 3 min, remaining 9 died 1 to 24 hours after the injection
60 mg/kg	6/6 survived 5 days	No immediate deaths, 12/12 mice died 2 to 24 hours after the injection

At the time these experiments were carried out the blood concentrations of paludrine had not been measured, and it was thought possible that the delayed deaths were caused by unusually prolonged retention of the drug in the blood, and therefore that an additive effect might be produced by further intravenous injections

The idea was tested by giving a second injection, after various intervals, of an amount (20 mg /kg.) that, by itself, produced very few deaths. Seventy-two mice were injected in the beginning, 12 were kept as controls and the remainder were divided into five further groups of 12 which were given a second injection 1, 3, 6, 24, and 48 hours respectively, after the first injection. The results are given in Table III. (The results of a second, similar experiment are given in parentheses in the Table)

TABLE III

MORTALITY IN MICE AFTER A SECOND INTRAVENOUS INJECTION OF 20 MG /KG PALUDRINE FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Figures in parentheses are the results of a second experiment

Group	Mortalities				Total dead after 5 days
	0-1 hr	1-5 hr	5-24 hr	24-48 hr	
I Control	—	—	—	1/12 (1/12)	1/12 (1/12)
2nd injection after					
II 1 hr	—	1/12	9/12	2/12	12/12
III 3 hr	—	—	9/12	—	9/12
IV 6 hr	—	—	11/12 (6/12)	—	11/12 (6/12)
V 24 hr	1/12	—	4/12 (1/12)	2/12 (2/12)	7/12 (3/12)
VI 48 hr	—	—	—	1/12	1/12

In mice, therefore, the second injection clearly exerts an additive effect. Similarly, a second injection of paludrine into rats also produces an additive toxic effect (Table IV), and in them, too, its parenteral injection is associated with delayed deaths. On the other hand, in chicks which have received paludrine intravenously, deaths occur within about 15 min or not at all, and in them a second injection does not produce an additive effect.

TABLE IV

MORTALITY IN RATS AFTER A SECOND INTRAVENOUS INJECTION OF 25 MG /KG PALUDRINE FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Group	Mortalities					Total Deaths
	0-1 hr	1-4 hr	4-8 hr	8-24 hr	24-48 hr	
I Control	—	—	—	—	—	0/6
2nd injection after						
II 1 hr	2/6	—	1/6	3/6	—	6/6
III 3 hr (1/6 dead before 2nd injection)	—	—	2/5	2/5	—	4/5
IV 6 hr	—	—	2/6	3/6	—	5/6
V 24 hr	—	—	—	—	—	0/6

Although these results tended to support the suggestion that paludrine might be highly persistent in rats and mice, measurements of blood concentrations soon disproved this, and we now find it difficult to believe that delayed deaths or additive effects are due to unchanged paludrine. We are therefore searching for a metabolite in the hope that the properties of the latter may provide an explanation.

Chronic toxicity

(a) *In mice*—Two types of experiment were done. In the first, mice weighing 18 to 22 g. were arranged in groups of 10 and dosed twice daily for 5 days with the test solutions. The LD₅₀ in this experiment is about 25 mg./kg., at 12.5 mg./kg. no animals die, at 50 mg./kg. they all die.

In the second type, young mice weighing 14 to 16 g. were dosed twice daily for 14 days. Growth appeared normal amongst those receiving 12.5 mg./kg., deaths occurred at higher doses.

(b) *In rats*—Newly weaned rats, weighing about 40 g., and selected from as few litters as possible, were arranged in groups of 10. Sexes and litter mates were distributed equally among the various groups. Food (standard cubes made to a formula of the Rowett Institute) was given to the animals immediately after the daily weighing at 10 a.m., water was always available.

The growth of rats for the first few weeks after weaning is linear and, with careful matching, all the groups in experiments such as the ones being described can be made to follow the same straight line. Seven days were allowed for the line to become established, and then treatment with paludrine was commenced. It was given orally, once daily.

It was found that a dose of 50 mg./kg. caused an immediate alteration in the slope of the growth curve, although not sufficient to reduce it to zero. Scattered deaths also occurred with this treatment. With a dose of 40 mg./kg. a slight deviation of the curve was caused sometimes immediately, sometimes later. With a dose of 30 mg./kg. growth was normal over the whole period of treatment (two months in some experiments) and there were no deaths.

Rats which died, and the survivors of all groups, were subjected to a pathological examination, but nothing of significance was found*. It is noteworthy that in none of these toxicity tests has any symptom been produced in any of the mammalian species that would lead one to suppose that the drug had affected the central nervous system. Chemical estimations confirm that the amount of drug which can be recovered from the brain of rats and rabbits is insignificant (see below and Spinks, 1947).

Blood concentrations†

The rat growth test just described is probably one of the most sensitive toxicity tests available in the laboratory and it was regarded as important to determine the concentrations of paludrine in the blood associated with the doses,

*We are indebted to Dr J. R. M. Innes for this information. The organs examined were brain, kidney, liver, pancreas, spleen, lung, intestine, and thyroid.

† All concentrations, whether in plasma, whole blood or tissue, are given as mg./l. or mg./kg. of the free base.

50 mg /kg and 30 mg /kg per day respectively, which delimited the toxic region. They were measured by the method of Spinks and Tottey (1946). Measurements on whole blood were made, in different experiments, after the first dose and after the seventh dose. At least three rats were used in the determination of each point. A curve for the concentrations reached on the seventh day is given in Fig. 1.

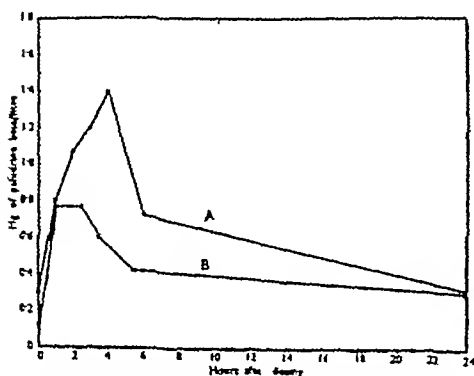


FIG 1—Concentrations of paludrine in the whole blood of rats after the seventh dose of 50 mg./kg. (A) and 30 mg./kg (B) once daily

The most interesting feature of the results is the fact that the blood concentration associated with a dose of 50 mg /kg once daily, which produces deaths in some rats, is comparatively low and has a peak of only about 1.4 mg /l. That such a concentration should be toxic in rats is of interest, because we believe it to be readily tolerated by human beings, 700 mg of paludrine have been administered twice daily in man with only mild toxic effects (Adams *et al*, 1945), while plasma concentra-

tions of about 0.5 mg /l have been recorded twelve hours after doses of 500 mg twice daily (Maegraith *et al*, 1946). The maximal concentration in the plasma following the latter dose was found to be about 0.7 mg /l in one subject, who, however, showed minimal concentrations rather lower than normal (Maegraith *et al*, private communication). Since the whole blood concentration in man is between 2 and 3 times the plasma concentration (Maegraith *et al*, 1946), it is reasonable to assume that blood concentrations between 1 mg and 1.5 mg /l are attained following the administration of 500 mg twice daily. The comparison can also be made on the basis of plasma concentrations. The maximal plasma concentration given in rats by the (toxic) dose of 50 mg /kg daily is 0.236 mg /l (Table V). Concentrations much higher than this have been frequently observed in man. A further point of difference between man and rat is the lower persistence of paludrine in the latter, illustrated by the low minimal concentrations, and by the fact that the concentrations determined after only one dose are very similar to those determined after 7 doses.

It now became important to determine the blood concentrations associated with chronic toxic effects in other species. We chose to examine the mouse and the chick because the mouse behaved like the rat in the matter of delayed deaths after parenteral injection, whereas the chick did not. Curves for whole blood concentrations in these two species are given in Figs. 2 and 3.

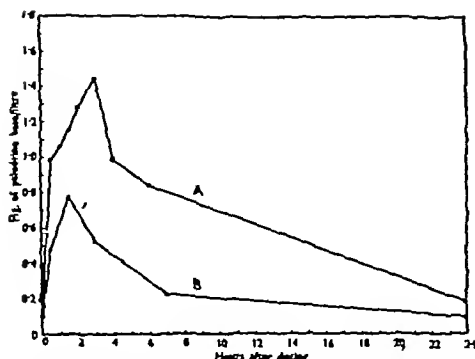


FIG 2.—Concentrations of paludrine in the whole blood of mice after the third dose of 30 mg/kg once daily (A) and the fifth dose of 12.5 mg/kg twice daily (B)

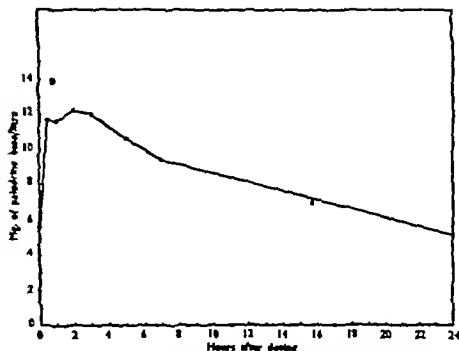


FIG 3.—Concentrations of paludrine in the whole blood of chicks after the fourth dose of 60 mg/kg. twice daily

The curves for mice were determined on one group receiving 12.5 mg/kg. twice daily, a treatment which is apparently harmless, and on another receiving 30 mg./kg once daily, a treatment which produces scattered deaths. In the first group measurements were made after the fifth dose, in the second after the third dose. It will be seen that there is a good parallelism between the concentrations toxic for mice and those toxic for rats, and that the general form of the curves is similar, the build up being negligible. It will also be apparent that the same dose given to mice and rats on a weight basis will produce higher concentrations in the mice.

Curves for chicks were obtained from animals receiving 60 mg./kg. twice daily. Scattered deaths occur with this regime although about 30 to 50 per cent of the animals will survive indefinitely treatment given for 5 days. Measurements were made after the fourth dose. It will be seen that the peak concentrations associated with a potential lethal effect in chicks are about 10 times as high as concentrations associated with lethal effects in rats and mice. Also the build up is considerable, a residue of about 5 mg./l. being left after the third and fourth doses. The contrast between chicks on the one hand, and rats and mice on the other, is also well shown by the results of other experiments in which only one dose of 50 mg./kg. was given to chicks. Peak concentrations of paludrine in the blood rose to 3–4 mg./l. and after 24 hours concentrations of 1.5 mg./l. were recorded.

DISCUSSION

Clearly a fundamental difference must exist between the metabolism of paludrine in rats and mice and its metabolism in chicks (and probably man). Certainly, the grosser aspects of distribution which can be measured chemically do not account for the differences in results. In all species examined so far the concentration of the drug in the plasma is about a third to a fifth that in whole blood, and the ratios between tissue and plasma concentrations (which vary from 10 to 100/1 depending on the tissue) are similar. We sought to emphasize the distinction between the chick and the rat, and the fact that the tissue distribution of the drug does not reveal any explanation of it, by comparing the concentrations found in the tissues of rats and chicks following the seventh dose of

50 mg /kg once daily This treatment is lethal for some rats, but tolerated by chicks The blood and tissues of 3 rats or 6 chicks were used at each time interval. The results are shown in Tables V and VI

TABLE V

DISTRIBUTION OF PALUDRINE IN RATS FOLLOWING THE SEVENTH ORAL DOSE OF 50 MG /KG ONCE DAILY

Time	mg base/l or kg in						
	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain
Before	0.403	0.0693	1.61	0.414	0.577	0.748	0
1 hour	0.802	0.0804	4.34	2.62	3.03	20.6	0
2 hours	1.07	0.154	11.2	4.69	9.14	32.4	trace
4 "	1.40	0.236	17.9	12.3	9.80	30.1	0
6 "	0.723	0.144	6.41	1.95	2.10	11.9	trace
24 "	0.320	0.0671	1.82	0.711	0.947	1.07	(0.123)

TABLE VI

DISTRIBUTION OF PALUDRINE IN CHICKS FOLLOWING THE SEVENTH ORAL DOSE OF 50 MG /KG ONCE DAILY

Time	mg base/l or kg in						
	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain
Before	3.25	1.02	72.4	25.9	92.6	45.3	11.5
1 hour	3.78	1.31	62.9	26.6	89.7	62.8	10.5
2 hours	8.15	2.84	121	73.8	214	136	19.4
4 "	7.59	2.58	117	57.6	246	104	17.5
6 "	5.91	2.14	103	40.9	177	95.1	18.7
24 "	2.95	0.809	75.6	12.4	53.9	31.6	15.1

Although the treatment is tolerated by chicks, but fatal for some rats, the drug concentrations are uniformly higher in the chicks. It would seem, too, that paludrine reaches the brain more readily in the chick than in the rat, a point which is of interest because, so far as we are aware, such a species difference has not been demonstrated for any other drug. However, this difference would hardly seem to have any bearing on the high blood concentrations in the chick or the delayed deaths in mice and rats. Surveying the results of all the experiments, we have come to the conclusion that the simplest explanation of them is to postulate that paludrine, in mice and rats, is metabolized to a substance persistent in the body and more toxic than the drug itself, in chicks, and probably in man, the metabolism is either different qualitatively or, if it is similar, the degree of degradation to the toxic substance is much less. The relevant facts can be summarized as follows:

1. Equivalent doses of paludrine give higher concentrations, and the drug is more persistent, in chicks than in mice and rats. On the other hand, it is more

toxic for mice and rats than for chicks. On the evidence so far available, it is probable that man behaves like the chick rather than like the rat or mouse.

2 Although paludrine appears to be removed so readily from the blood of mice and rats, delayed deaths may occur in both species and, after parenteral administration, an additive toxic effect can be produced by a second injection given even 24 hours after the first.

3 The tissue distribution of the drug in its grosser aspects does not account for the differences in susceptibility between chicks and rats. Concentrations are uniformly higher in the chicks.

4 The distribution of 4430 (an N_2 -methyl derivative of paludrine) in the body is similar to that of paludrine (Spinks, 1946, 1947), but delayed deaths are not associated with its injection into animals.

5 Recoveries of paludrine from the faeces and urine of rats (and rabbits) are low, usually less than 30 per cent of the dose (Spinks, 1947), which contrasts markedly with what obtains in man, where they are much higher, often up to 60 per cent (Macgrath *et al*, 1946).

SUMMARY

1 Measurements of the toxicity of paludrine for mice, rats, rabbits, and chicks are given.

2 Delayed deaths follow the intravenous injection of paludrine into mice and rats, and it is noteworthy that an additive toxic effect can be obtained by a second intravenous injection given many hours after the first. Delayed deaths do not follow the intravenous injection of the drug into chicks, and in these animals an additive toxic effect is not produced by a second injection.

3 Measurements of the concentration of paludrine in the blood of mice, rats, and chicks under various treatments showed that chicks tolerate much higher concentrations of the drug in the body than do mice and rats.

4 Gross measurements of the drug in the organs of chicks and rats on a similar treatment (50 mg/kg once daily) did not reveal differences sufficient to account for the difference in tolerance. Because of this, and in the light of other evidence which is presented, it is suggested that the metabolism of paludrine in chicks is different from what it is in rats and mice. The facts would be explained if paludrine, in mice and rats, were degraded in large measure to a substance more toxic than the drug itself.

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pA, A NEW SCALE FOR THE MEASUREMENT OF DRUG ANTAGONISM

BY

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When the activity of a drug can be expressed in terms of a stable standard which does not differ from it qualitatively, there is no difficulty in getting reproducible results, since all the assays tend to give the same answer, and any degree of accuracy can be attained provided that the experiment is repeated sufficiently often. When, however, the activity of a new drug or drug antagonist has to be defined in terms either of some other drug or of some of its own effects, the results are not equally reproducible since the apparent activity varies in successive experiments, even though conditions are kept as constant as possible. The difficulty of making results of one laboratory available to another is aggravated by the multiplicity of methods used and frequently by the lack of information of their variability, this applies particularly to methods of expressing drug antagonism.

It would obviously be of advantage if some common method of expressing drug antagonism could be agreed upon. In the present paper it is proposed to introduce a new measure of drug antagonism, pA, based on a suggestion made originally by Clark and Raventos (1937). Apparatus and methods are described for determining pA accurately on the guinea-pig's ileum, and the activity of several known antagonists of histamine and acetylcholine has been measured in terms of pA. The variability of the pA measure has been estimated and methods are discussed of obtaining reproducible results in the most economical way.

APPARATUS

All the experiments were done on the isolated ileum of the guinea-pig. The apparatus used for assaying drug antagonists is shown in Fig. 1. It consists essentially of a gut bath which can communicate with two alternative systems, one of which is filled with ordinary Tyrode solution and the other with Tyrode solution containing the antagonistic drug, the latter solution can be replaced in the course of the experiment without interfering with the assay.

All the operations involved in an assay, except the injection of the drug, are performed automatically. The principle of the method has been described before (Schild, 1946). "Telephone relays are converted to compress rubber tubing. When the relays are activated the rubber tubing is decompressed and fluid is allowed to flow. These relays control the emptying and filling and the adjustment of fluid level of an isolated organ bath. They are activated at regular time intervals through a telephone unselector which makes 12 successive

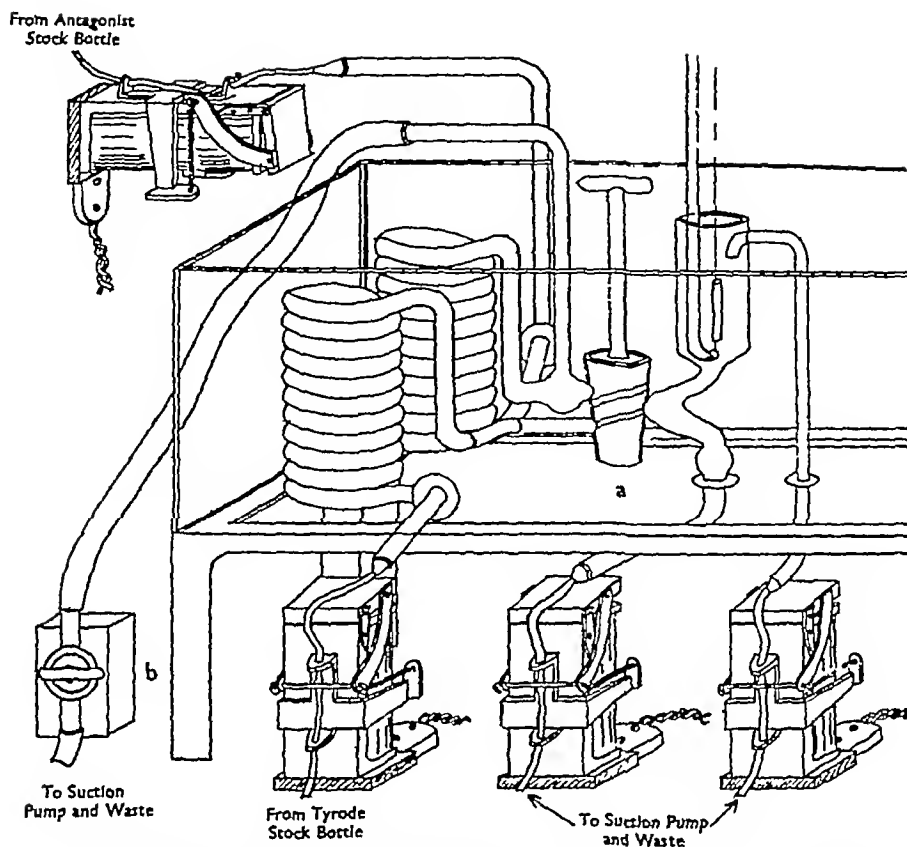


FIG 1—Apparatus for assaying drug antagonists Two stock bottles provide the inflow, one filled with Tyrode solution, and the other with a Tyrode solution of the antagonist drug. All the outflows are connected to a water suction pump. The electro magnets compressing rubber tubing are automatically operated through the selector circuit shown in Fig. 2. They are standard P O telephone relays of 3,000–6,000 ohms resistance operated directly from D.C. mains, with a special armature made in our workshop. The movement of the armature is controlled by two coiled springs which tend to compress rubber tubing of 2×4 mm diameter by means of a thin brass rod.

contacts in a cycle. The duration of each contact is usually 15 sec., thus producing a cycle of 3 min. The selector also controls the movements of the drum and a light signal to time the injection of drugs. A diagram of the selector circuit is shown in Fig. 2.*

The present apparatus differs in the following respects from the one previously described.

1. An additional inflow relay is provided to control the inflow of the antagonistic solution. The two inflow relays are activated simultaneously, allowing one of the two solutions to flow into the bath according to the position of the 3-way tap. By means of

*All the component parts of the automatic apparatus, including A.C. mains-operated electro-magnets compressing rubber tubing, and A.C.-operated 12-step selectors, are now made by Messrs. Londex, Ltd.

switch D (Fig. 2) the selector can be short-circuited and the inflow relays activated directly. If the solution in the system containing the antagonistic drug is to be replaced, the 3-way tap *a* (Fig. 1) is turned into the null position, switch D is activated, and tap *b* is opened, allowing the solution to drain into the exhaust.

2. Switches A, B, and C control certain alternative arrangements of the cycle. By means of switches A and B the gut bath may be emptied and refilled twice in succession instead of only once. By means of switch C the signal may be advanced by 15 sec., permitting a longer period of contact between drug and tissue. Table I shows the automatic operations performed in a complete cycle together with the possible alternative arrangements.

3 The outflows are operated by suction.

4 A new type of adjustment for telephone relays has been used to compress rubber tubing. The adjustment is somewhat more complicated than the one previously described, but it is more efficient and stable. It is illustrated in Fig. 1

5 Two gut baths have been operated simultaneously. The baths were contained in a large thermostatically controlled tank, stirred by a circulating water-pump. As a rule a lag period of 30 sec was maintained between the two cycles, tracings being recorded on two independent drums situated at opposite ends of the tank.

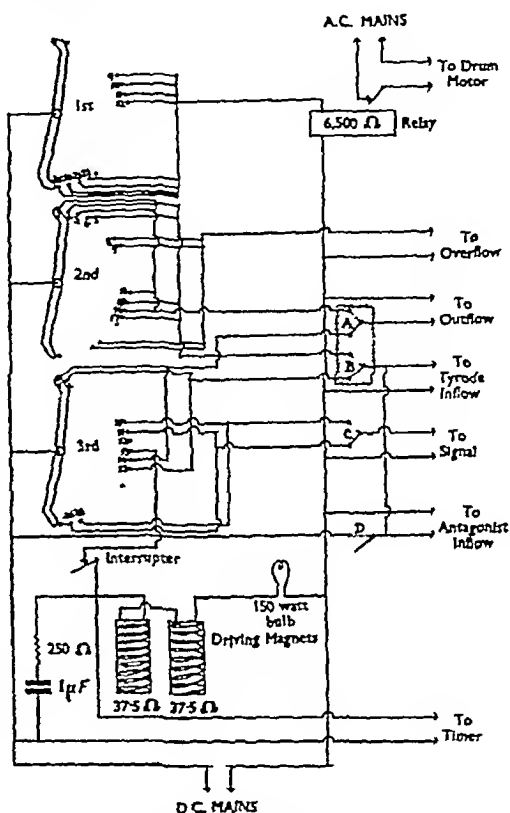


FIG. 2.—Diagram of selector circuit for one muscle bath.

TABLE I
STEPS IN CYCLE

Relays	1	2	3	4	5	6	7	8	9	10	11	12
Outflow { usual alternative	+		+									
Inflow { usual alternative		+		+								
Signal { usual alternative										+	+	
Overflow								+	+			
Drum									+	+	+	+

The writing lever used in these experiments is illustrated in Fig. 3

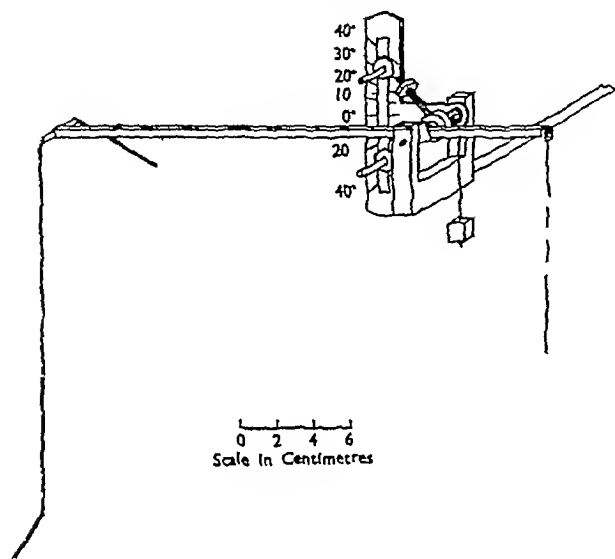


FIG 3—Approximately linear and isotonic frontal writing lever

The main purpose of adding the antagonistic drug to the bath fluid before it reaches the gut, instead of adding it in the usual way by injection into the muscle bath, is to prevent the gut coming into contact with pure Tyrode solution during the period of replacement of the bath fluid when the effect of an antagonist on successive contractions is being investigated. The present method is thus particularly suitable for investigating the effect of antagonists in relation to time, whilst both methods are suitable for determining the effect of antagonists on a single contraction. The old method is most suited for preliminary experiments and for comparative measurements.

The purpose of the long writing point used on the frontal writing lever is to ensure that the relation between shortening of the gut and effect on the drum should be linear. This is especially important when the effects are measured in terms of a maximum contraction. The errors obtained with a shorter writing point are, however, usually not excessive, unless a very short writing point is used or the angle of excursion of the lever becomes greater than about 30° from the horizontal, this may be prevented by means of two adjustable stops limiting the excursion of the lever as shown in Fig. 3. The errors may be calculated from the formula given in a previous communication (Schild, 1944). The lever can be made practically isotonic by means of the screw adjustment shown on the pivot. The adjustment may be tested by suspending an appropriate weight at the point of attachment of the thread. The lever should then balance in every position in which it is likely to be used. The tension exerted by the lever in these experiments was of 600–800 mg.

The following antagonist drugs were used in these experiments

Neoantergan, or N-p-methoxybenzyl-N-dimethylaminoethyl- α -aminopyridine acid maleate (Bovet, Horclois, and Walther, 1944)

Benadryl, or dimethylaminoethyl benzhydriol ether hydrochloride (Loew, Kaiser, and Moore, 1945)

It may be asked to what extent these modifications of the usual Burn-Dale isolated organ bath are essential for these experiments

The purpose of the automatic apparatus is to ensure constant time intervals and bath volumes, to enable more than one assay to be performed at the same time and in general to enable the experimenter to divert his attention from servicing the bath and drum. In practice the automatic apparatus has been found very useful, but it is obviously not essential for carrying out these tests since all the operations can be performed by hand

Pethidine, or ethyl 4-phenyl-1-methylpiperidine-4-carboxylate hydrochloride (Schaumann, 1940)

Atropine sulphate.

THE pA SCALE

pA_x is defined as the negative logarithm to base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose. Thus, if the presence of a concentration of 10^{-5} molar pethidine in the bath fluid reduces the effect of 2 μ g histamine to that produced, in the absence of pethidine, by 1 μ g histamine, pA_2 pethidine-histamine = 5.8

It is obvious that a constant of this nature can refer only to a given drug-antagonist pair acting on a definite pharmacological preparation, e.g., the guinea-pig's ileum, and that a representative pA value must be the mean of several individual determinations. pA values are dependent on the length of contact between antagonistic drug and tissue, but they are apparently independent of the absolute concentrations of the active drug used. These points will be discussed in detail later.

Method of determining pA_2 .—The principle of the method consists in finding two concentrations of the antagonistic drug such that one will reduce the effect of a double dose of the active drug to slightly less and the other to slightly more than the effect of a single dose. The concentration corresponding to pA_2 is then computed by interpolation on a logarithmic scale.

The following results justify to some extent the use of a logarithmic scale for interpolation. In a series of pA_2 determinations a third concentration of antagonist was added to test for linearity between log concentration of antagonist and effect. The points, plotted on a logarithmic scale in Fig. 4, each point representing the mean of several determinations, fall on approximately straight lines.

In order to get reliable results a constant submaximal response to the stimulant drug must be produced before addition of the antagonist, 10–20 preliminary contractions may be required to achieve this. At this point the muscle chamber

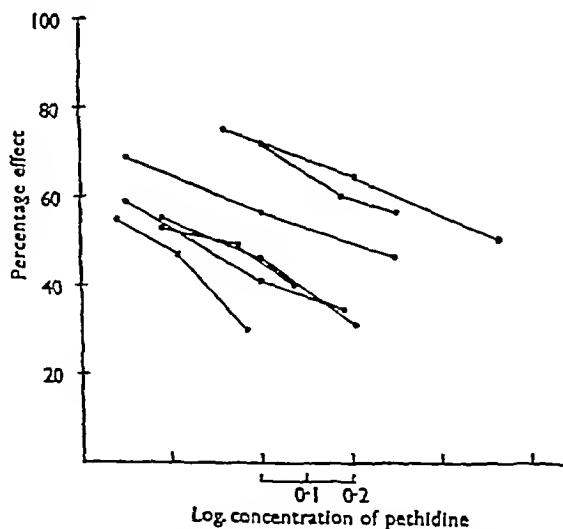


FIG. 4—Guinea-pig ileum. Pethidine-histamine. Approximately linear relation between log concentration of pethidine and reduction of histamine effect.

is joined to the system containing a Tyrode solution of the antagonistic drug, which now replaces Tyrode solution when the bath fluid is changed. At the next injection the dose of active drug is doubled. Injections of a double dose of active drug in the presence of the antagonist are continued for several periods (usually five periods) in order to observe whether the effect of the antagonist increases with increasing length of contact. Eventually the bath fluid is switched back to Tyrode solution to test for persistence of antagonistic effect and the assay is concluded by producing a series of maximal effects. A second concentration of antagonist is investigated in the same way, usually on a fresh piece of gut, and the results, expressed in terms of the maximal contraction, are used for interpolating pA_2 values after various periods of contact with the antagonist.

As in these experiments the automatic apparatus was set to produce intervals of 3 min. between injections and pauses of 2 min. between completion of change of bath fluid and the next injection, the first pA measurement was made when the antagonistic drug had been in contact with the tissue for 2 min. and further determinations at 3 min. intervals. During this period the tissue never ceases to be in contact with the antagonistic drug which is contained in the bath fluid itself, and an even flow of stimulation at constant intervals is maintained as the period of contact with the antagonist is gradually increased.

Although tedious, the use of a fresh piece of gut for each concentration of the antagonist has been found to be the most satisfactory procedure when the effect of the antagonist is persistent. In our experience, variations in sensitivity to the action of antagonists of different segments of the same gut are remarkably small and are not correlated with variations in sensitivity to the stimulant drug, provided that pieces which are obviously damaged and insensitive are rejected. Although it is advantageous to use two pieces of gut simultaneously, this is not essential, since the guinea-pig ileum does not seem to alter appreciably in sensitivity if left in clear Tyrode solution at room temperature for several hours.

Fig 5 (p 205) illustrates two complete pA_2 determinations done on six segments of the same gut. The object was to determine pA_2 values after a short period of 2 min. and after a long period of 14 min. contact with the antagonist. The following two examples, quoted from the experiment shown in Fig 5, illustrate the method of computing pA_2 .

pA_2 , benadryl-histamine (2 min. contact)—After 2 min. contact between muscle and antagonistic drug a double dose of histamine in $10^{-7.44}$ molar benadryl (1 300 million) produces an effect which is 7 per cent (of the maximal effect) greater, and a double dose of histamine in $10^{-7.44}$ molar benadryl (1 100 million) an effect 11 per cent smaller, than that produced by a single dose of histamine in Tyrode. By interpolation the molar concentration of benadryl which would just reduce the effect of a double dose of histamine to that of a single dose is $10^{-7.73}$ (1 195 million). Hence pA_2 (2 min. contact) = 7.75.

pA_2 , neoantergan-histamine (14 min. contact)—When the antagonist has been in contact with the muscle for 14 min. (5th injection in presence of antagonist) a double dose of

histamine in $10^{-3.33}$ molar neoantergan (1 9,000 million) produces an effect 7 per cent greater, and the same dose in $10^{-3.66}$ molar neoantergan (1 3,000 million) an effect 10 per cent smaller, than a single dose of histamine in Tyrode By interpolation pA_2 (14 min contact)=9.36

Independence of pA and concentration of antagonist—Since the absolute concentration of the active drug does not enter into the definition of pA , it was of interest to find out whether the depression produced by a certain concentration of the antagonist was, in fact, independent of the concentration of active drug used Although this is generally accepted as true (Gaddum, 1937), it seemed worth investigating the point, using a properly randomized experimental design which could be statistically analysed

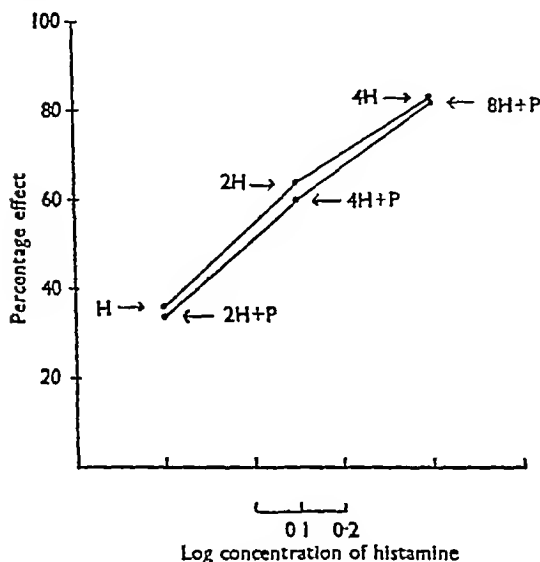


FIG 6—Effect of a constant dose of pethidine (P) on contraction produced by various concentrations of histamine (H) The depressor effect of the antagonist is independent of the contraction produced by the active drug Upper curve histamine alone. Lower curve double dose of histamine+pethidine.

of the histamine effect by pethidine was approximately the same There was no statistically significant deviation from parallelism between the two curves Similar results were obtained in the other experiments.

ANTAGONISM OF NEOANTERGAN, BENADRYL, PETHIDINE, AND ATROPINE TOWARDS HISTAMINE AND ACETYLCHOLINE

The activity and relative specificity of these antagonists of histamine and acetylcholine can be conveniently summarized in terms of pA . In Fig. 7 the

The experiments were carried out as follows Several doses of the active drug alone and of the active drug plus antagonist were administered If the antagonist produced the same amount of depression at each concentration of the active drug, the concentration-action curves in the presence and the absence of the antagonist should be parallel Doses were administered repeatedly in a random order, and the results were analysed by means of analysis of variance for statistically significant deviations from parallelism

Five such experiments were performed, three with pethidine-histamine, one with atropine-acetylcholine, and one with pethidine-acetylcholine One of the experiments is illustrated in Fig. 6, each point on the curve representing the mean of four determinations It will be seen that at each level the reduction

two vertical lines represent pA_2 scales for antagonists of histamine and acetylcholine. The activity of an antagonist towards these drugs is indicated by its position on the scale, one scale division corresponding to a tenfold difference in activity. Points on the two scales referring to the same antagonist are joined. If an antagonistic drug does not discriminate between two active drugs, the line joining the scales is horizontal, if it discriminates sharply the line is steep, as with neoantergan and atropine. Neoantergan is the most discriminating of the four antagonists, being 40,000 times as active against histamine as it is against acetylcholine. Atropine is 1,000 times as active against acetylcholine as it is against histamine. Pethidine, on the other hand, discriminates hardly at all between the two.

A single pA value is not sufficient to characterize an antagonist fully. To describe fully the relation between a given antagonist and an active drug it would be necessary to state completely both the time-action and the concentration-action relations of the system. Short of this, four characteristic pA values have been selected in the present study which together give some indication of the change of activity of an antagonist with concentration and with time. The

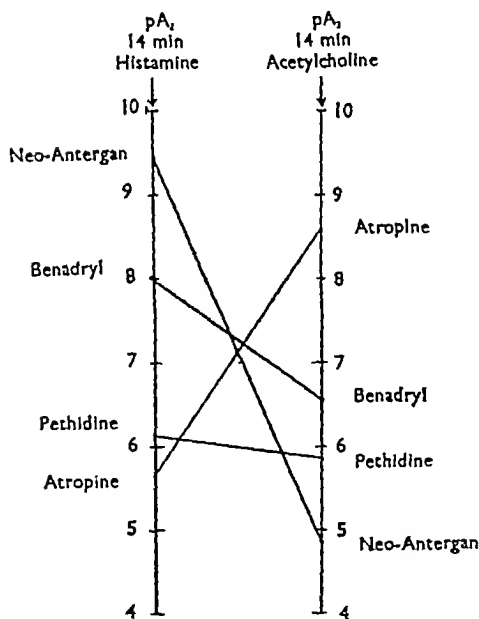


FIG 7— pA_2 scales of histamine and acetylcholine. At the time of measurement the antagonist had been in contact with the tissue for 14 min. The results may be taken to represent approximately equilibrium conditions.

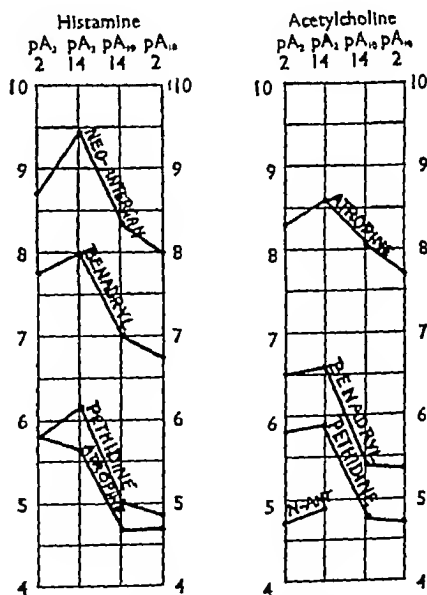


FIG 8—Each antagonist of histamine and acetylcholine is characterized by four pA values. Note qualitative differences between antagonists with regard to increase of action with time and pA_2 - pA_{100} difference.

following four pA values have been selected for this purpose the pA_2 values after 2 and 14 min contact and the pA_{10} values after the same two periods of contact.

A 14 min. period of contact with the antagonist (corresponding to five consecutive contractions in the presence of an antagonistic drug) has been arbitrarily chosen as representing approximately equilibrium conditions, since at that time there is usually not much further increase of depression. It would, of course, be preferable always to establish true equilibrium conditions, but it was found that in some instances the effect of the antagonist appeared to increase indefinitely, when this happens it becomes difficult to distinguish between the effect of the antagonistic drug and that of a spontaneous deterioration of the preparation, and true equilibrium conditions cannot be established.

A convenient method of summarizing the four pA values is shown below

<i>Atropine—Acetylcholine</i>				<i>Pethidine—Acetylcholine</i>			
	pA_2	pA_{10}	$pA_2 - pA_{10}$		pA_2	pA_{10}	$pA_2 - pA_{10}$
2'	8.37	7.72	0.65	2	5.79	4.69	1.10
14'	8.77	8.05	0.72	14	5.89	4.71	1.18
14'-2	0.40	0.33		14-2	0.10	0.02	

The data are taken from three experiments in which pethidine was tested against acetylcholine and three further experiments in which atropine was the antagonist, each experiment being complete in the sense that all the four pA values were determined in the course of a single assay. When the data are tabulated in this manner the row differences provide a measure of the steepness of the concentration-action curve, and the column differences indicate changes of activity with time. Pethidine and atropine differ in both these respects. The row differences are of the order of 0.7 for atropine and of 1.1 for pethidine, indicating that in order to compensate for a fivefold rise of acetylcholine the concentration of atropine has to be raised fivefold and that of pethidine thirteenfold. The difference is statistically significant. The differences in the columns show that after 14 min contact the effect of atropine is over twice that after 2 min contact, whilst the effect of pethidine increases hardly at all during this period.

The difference between pA_2 and pA_{10} provides a quantitative test for the hypothesis that antagonists compete with drugs for receptors according to a simple mass action relation. It can be shown that the mass action equation as developed by Gaddum (1937) for a first order reaction requires a ninefold increase of antagonist corresponding to a fivefold increase of active drug between pA_2 and pA_{10} . Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation.

The results of pA determinations are summarized in Table II. Each drug-antagonist pair is characterized by four pA values. The figures given are mean values, the total number of individual determinations and their standard deviation being indicated in the Table. Fig 8 shows some of the differences between antagonists as revealed by the measurement of four pA values for each. Apart from differences in their general activity, antagonists also show characteristic differences in time-action and concentration-action relations. The lines joining the outer scales to the two inner scales represent increases of activity with time, these are greatest in the two most active antagonists. The lines joining the two inner scales indicate differences between pA_2 and pA_{10} (at approximately equilibrium conditions). This difference is smallest with the pair atropine-acetylcholine, which provides the only instance of straight proportionality between concentration of drug and antagonist. In all other cases a tenfold or greater increase of antagonist concentration is required to balance a fivefold increase of drug concentration.

One of the most interesting findings has been a complete lack of correlation in the behaviour of the same antagonist when tested against two different drugs. This applies both to time-action and concentration-action relations. Thus, the effect of neoantergan after the first 2 min contact increases little further against acetylcholine, but continues to increase steeply against histamine. As for concentration-action curves, atropine has a steep concentration-action curve against acetylcholine and a relatively flat one against histamine. These results suggest that when the same antagonist antagonizes two different drugs the mechanisms involved may be quite different.

After-effects of antagonists—The rate of recovery after the antagonist has been removed from the bath varies in much the same way as the rate of development of the effects, and here again the same antagonist may be persistent when assayed against histamine and non-persistent against acetylcholine and *vice versa*. Fig 5 shows examples of slow (neoantergan) and quick (benadryl) recovery from antagonists on the same preparation.

A curious after-effect which occurred at times, especially with neoantergan, is shown in Fig 9 (p 205). The maximum depression of the effect of histamine did not take place in the presence of neoantergan, but shortly after it had been removed from the bath, as if the act of washing out the antagonist had further increased its effect.

VARIABILITY OF pA DETERMINATIONS

Determinations of pA_2 pethidine-histamine were made in 19 different experiments spread over a period of over one year. The results were distributed as shown in Fig 10. The variations of pA_2 indicate a 3.2-fold (or, omitting one result, 2.4-fold) variation in the sensitivity of the tissue. The shape of the

distribution curve suggests sampling from a non-homogeneous population. In 10 of these experiments two or more pA_2 determinations were made on the same gut and consequently variation between animals could be compared with variation in successive tests on the same animal by means of Fisher's Z test. The resulting Z value was highly significant, suggesting that there is true variation between guinea-pigs in their sensitivity to antagonists

TABLE II
RESULTS OF pA DETERMINATIONS

The total number of individual determinations is given in parentheses and σ is the standard deviation.

Active drug	Antagonist	pA_2		pA_{10}	
		2 min	14 min	2 min.	14 min
Acetylcholine	Atropine	8.27 (11) $\sigma = 0.11$	8.61 (11) $\sigma = 0.15$	7.72 (3) $\sigma = 0.07$	8.05 (3) $\sigma = 0.13$
	Benadryl	6.49 (3) $\sigma = 0.07$	6.57 (3) $\sigma = 0.09$	5.36 (2) $\sigma = 0.06$	5.4 (2) $\sigma = 0.04$
	Pethidine	5.79 (3) $\sigma = 0.07$	5.84 (4) $\sigma = 0.14$	4.7 (4) $\sigma = 0.05$	4.76 (5) $\sigma = 0.15$
	Neoantergan	4.71 (2) $\sigma = 0.06$	4.86 (2) $\sigma = 0.09$		
Histamine	Neoantergan	8.71 (4) $\sigma = 0.15$	9.46 (5) $\sigma = 0.22$	7.99 (1)	8.36 (1)
	Benadryl	7.75 (8) $\sigma = 0.1$	8.02 (9) $\sigma = 0.28$	6.74 (4) $\sigma = 0.11$	7.02 (4) $\sigma = 0.31$
	Pethidine	5.78 (19) $\sigma = 0.14$	6.13 (5) $\sigma = 0.46$	4.84 (5) $\sigma = 0.17$	5.0 (5) $\sigma = 0.21$
	Atropine	5.73 (3) $\sigma = 0.22$	5.64 (3) $\sigma = 0.18$	4.63 (3) $\sigma = 0.24$	4.6 (3) $\sigma = 0.24$

pA measurements are more variable after long periods of contact with the antagonist than after short periods. The following standard deviations summarize the variation encountered for the four different types of pA measurements

	Standard deviation	Degrees of freedom
pA_2 , 2 min. contact*	0.13	31
pA_{10} , 2 min. contact	0.14	15
pA_2 , 14 min. contact	0.25	34
pA_{10} , 14 min. contact	0.22	16

*Omitting pethidine-histamine.

The standard deviation is nearly twice as great after 14 min as after 2 min. This is somewhat surprising, since variation might be expected to become less as equilibrium conditions were approached. The increase in variability may be partly due to spontaneous changes in sensitivity of the preparation during the longer period of contact.

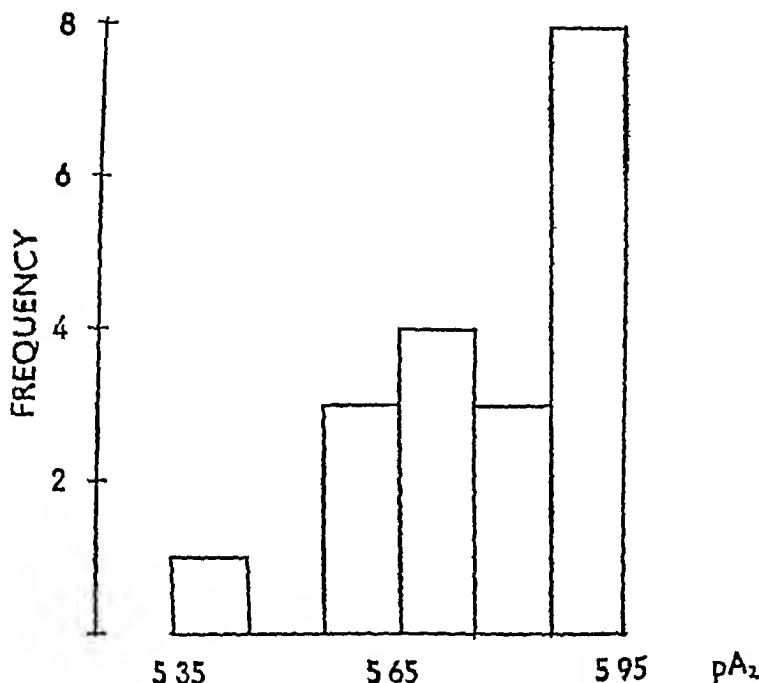


FIG. 10.—Frequency distribution of pA_2 values for pethidine-histamine obtained after 2 min. contact between drug and antagonist, 19 experiments.

THE USE OF COMPARATIVE ASSAYS FOR DETERMINING pA_2

It has been shown that animals vary in their sensitivity towards individual antagonists and that this variation is greater when the period of contact of antagonist with tissue is long than when it is short. It seemed possible that some of this variability might be eliminated in a comparative assay in which one antagonist was pitted against another antagonist. Such an experiment, involving repeated injections of each antagonist, might possibly be carried out on a single piece of gut so long as excessive depression by the antagonists was prevented by keeping the periods of contact short and by giving numerous "recovery" injections of the stimulant drug, after washing out the antagonist.

Six experiments were performed in which the action of pethidine towards histamine was compared with that of atropine on a single preparation. The experimental plan of these assays was similar to one previously used by the author (Schild, 1942) for histamine

assays In the histamine assay only two concentrations of the standard and two of the unknown are used These concentrations are given in a random order in successive "randomized groups" of four, and the results are eventually computed and analysed statistically on the assumption of a linear relation between log dose and effect In the present experiments the place of four concentrations of histamine is taken by two concentrations of each antagonist administered 2 min before the injection of a constant dose of histamine In addition several injections of histamine alone are given following each administration of the antagonist until the sensitivity to histamine has been apparently restored Fig. 11 shows parts of two such experiments In both cases a "randomized group" of four doses of antagonist is represented, a complete experiment consisting of several (2-4) such "random-

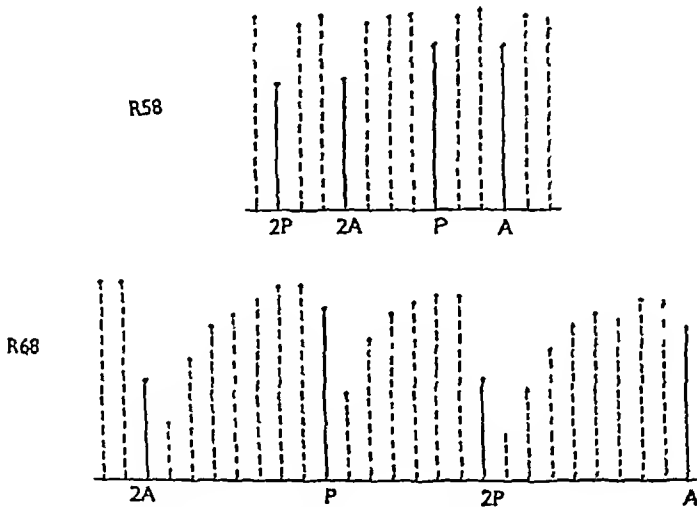


FIG 11—Comparisons of activity of pethidine and atropine towards histamine on a single piece of gut. Extracts from two such experiments The solid lines represent effects of histamine in the presence of an antagonist which has previously been in contact with the tissue for 2 min., the dotted lines the effects of the same dose of histamine alone administered at 3 min intervals until sensitivity is restored As far as possible approximately equally depressant doses of pethidine and atropine were used, pethidine being more than twice as active as atropine The final ratio of activity is calculated by assuming a common slope for the two antagonists and a linear relation between log concentration and effect.

ized groups In one of these experiments the antagonists produced little after-effect, in the other a prolonged depression necessitating many "recovery" injections In all the experiments statistical analysis showed satisfactory parallelism between atropine and pethidine slopes The results of these comparisons, which were done at various times and on different stocks, were remarkably constant The following figures of the logarithm of the ratio pethidine/atropine were obtained 0.33, 0.36, 0.37, 0.34, 0.3, 0.33

pA₂ atropine-histamine by direct and indirect method—pA values may be determined directly without reference to another antagonist, or indirectly, by measuring in a comparative assay, such as the one outlined above, the activity

of an unknown antagonist in relation to one whose pA is already established, and computing the unknown pA by adding to the known pA the logarithm of the ratio of molar activity of the two antagonists. Thus

$$pA_2 \text{ (atropine-histamine, 2 min)} = pA_2 \text{ (pethidine-histamine, 2 min)} - 0.34 \\ = 5.78 - 0.34 = 5.44$$

This method would seem to be economical provided that the results agree with those of direct determinations, this, however, is not entirely the case. There is a slight discrepancy between direct and indirect pA determinations which suggests that, although in comparing the activity of antagonists on a single piece of gut a 2 min. period of contact was adhered to, the gut responded in fact as if the period of contact had been longer. Indeed, the results agree better with direct pA_2 determinations done after 14 min. contact, as the following data show

	pA_2 (2 min contact)	Comparison on same gut (2 min. contact)	pA_2 (14 min contact)
Pethidine-histamine	5.78		6.13
Atropine-histamine	5.73		5.64
Log ratio $\frac{\text{pethidine}}{\text{atropine}}$	0.05	0.34	0.49

These results might be explained by assuming that in comparing antagonists on a single preparation they are never completely removed in spite of repeated washings.

In comparing two antagonists on the same gut the result may be vitiated by certain systematic errors unless care is taken that the effect of a previous dose has subsided at the time the next dose is added, especially when the two antagonists have different modes of action. The following example illustrates the point.

Antagonist (a) is to be compared with antagonist (b), their effects are additive but (a) has a persistent action and (b) a readily reversible action. When (a) is added to the bath some of its effect persists after it has been removed. If a depression is produced by (a) and matched at the next injection by a depression produced by (b) the real effect of the latter, acting as it does on a depressed preparation, is less than it appears. At the next injection the same concentration of (a) reproduces its old action, the effect of (b) having worn off.

Comparison of antagonists on separate pieces of the same gut—In these experiments direct pA determinations on two different antagonists were done simultaneously using a separate piece of gut for each antagonist and each concentration, all the segments being taken from the same guinea-pig. Variability between guinea-pigs was thus eliminated.

An experiment of this kind comparing the action of neoantergan and benadryl towards histamine is illustrated in Fig. 5. The results of this and two further similar experiments are shown in Table III.

The following points may be noted

(i) The difference between pA_2 values at 2 min. contact is remarkably constant, more constant than the absolute values. This confirms previous conclusions that the gut varies simultaneously in sensitivity towards different antagonists.

(ii) Increase of activity with time (pA_2 14 min $-pA_2$ 2 min) varies, but it is in each case considerably greater with neoantergan than with benadryl

(iii) At 14 min pA_2 values and their differences vary more than at 2 min This confirms previous conclusions

In conclusion it may be said that comparative measurements definitely eliminate a certain amount of variation Comparisons on a single piece of gut

TABLE III

pA_2 NEOANTERGAN-HISTAMINE AND BENADRYL-HISTAMINE DETERMINED IN THE SAME EXPERIMENT USING A SEPARATE PIECE OF ILEUM FOR EACH CONCENTRATION OF ANTAGONIST

		pA_2 values neoantergan benadryl		pA_2 neoantergan — pA_2 benadryl
R.205	2'	8.91	7.94	0.97
	14'	9.76	8.35	1.41
	14'-2'	0.85	0.41	
R.206	2'	8.61	7.66	0.95
	14'	9.60	7.78	1.82
	14'-2'	0.99	0.12	
R.207	2'	8.65	7.75	0.90
	14'	9.36	8.26	1.10
	14'-2'	0.71	0.51	

are efficient in the sense of allowing many determinations to be made in a relatively short time, but they may be affected by systematic errors owing to persistence of antagonistic effects. Comparisons on separate pieces of gut are laborious but free from objections and give a more complete picture, since the time factor may be taken into account.

DISCUSSION

Clark and Raventos (1937) proposed to use as a measure of activity of an antagonist the concentration which would neutralize the effect of a tenfold increase of active drug One of the chief merits of this measure is that, being a null measure, and involving no change in response, it is independent of the method of experimentation used, and yields results on different preparations which are directly comparable

pA_2 is based on the same idea as the measure adopted by Clark and Raventos, but it is expressed in a rather more convenient form. It may be used to define the activity and specificity of an antagonist, its time-action relations and the trend of its concentration-action curve pA_2 values are additive, for instance, in order to express the total activity of a compound against both histamine and acetylcholine, the respective pA_2 values may be added together

The activity of an antagonistic drug may be expressed in one of two ways, by reference to another antagonist or by some measurement not involving a comparison with another drug. Since the activity of a drug cannot as a rule be expressed accurately in terms of another drug which is qualitatively different, the comparative method gives an incomplete and often misleading picture of the activity of an antagonist. Moreover, unless a definite common standard has been agreed upon, results from different laboratories are difficult to correlate. Comparisons may nevertheless be useful in practice owing to their greater economy of time and effort, especially when a series of related compounds is being investigated within the same laboratory. This question has been discussed in detail above.

The real test of the usefulness of a measure of drug antagonism is whether the results can be reproduced in another laboratory. Variations occurring within the same laboratory can be overcome by repeating the experiment and by random selection of experimental animals, but systematic variations between different laboratories present a more difficult problem. At present it is not known whether pA will be affected by such systematic variations or whether results in different laboratories will fall within the range of variations obtained with a highly mixed stock of animals in an individual laboratory. If important variations in sensitivity between laboratories should occur, one way of eliminating them would be to use in this type of work a homogeneous strain of guinea-pigs, available to all the laboratories concerned. Failing this, it might become necessary to fall back on the method of establishing differences of pA between antagonists rather than absolute pA values in the hope that the former would be less subject to laboratory variation than the latter. The use of an agreed measure of drug antagonism should, at least, make it easier to detect the occurrence and extent of such variations.

SUMMARY

1 Clark and Raventos (1937) suggested a method of estimating antagonistic power in terms of "the concentration of antagonist which altered by a selected proportion (e.g., tenfold) the concentration of an active drug needed to produce a selected effect." The negative logarithm of this (molar) concentration has been termed pA_x , where x is the proportion selected. On the guinea-pig's ileum the value of pA appears to be independent of the degree of contraction produced by the active drug. Methods are described for determining the value of pA .

2 pA is a statistical constant. To obtain a representative value of pA for a given tissue, antagonist, and active drug, the mean of a random sample of determinations on different individual animals must be determined. pA values were found to vary by 0.4 to 0.5 of a unit when the period of contact of the muscle with the antagonist was short, at long periods of contact variability was increased. Variability may be reduced by comparing the activity of one

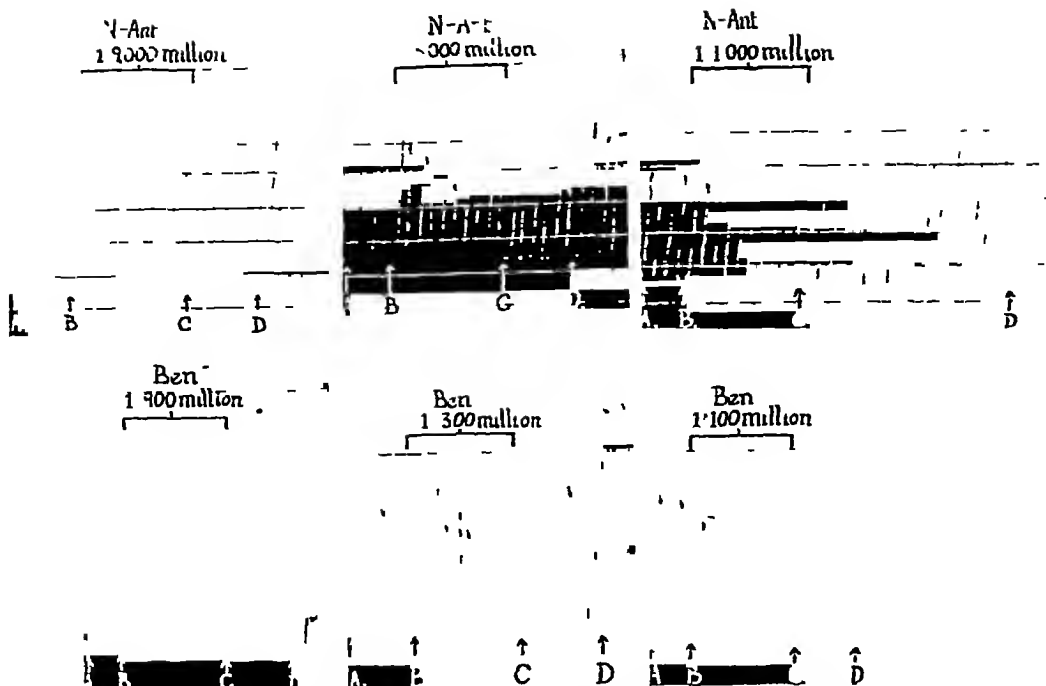
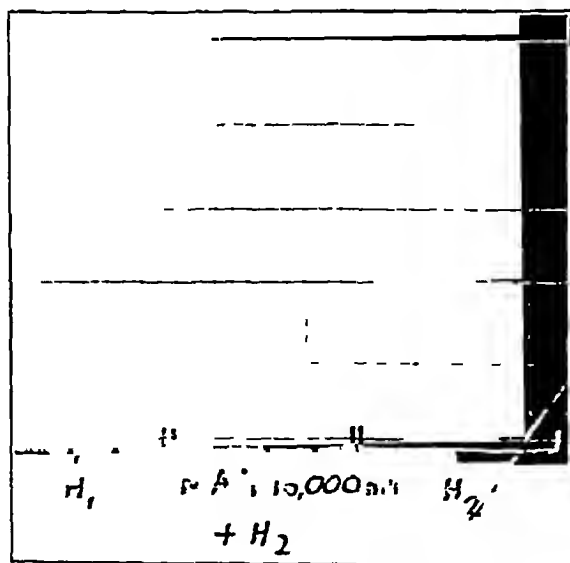


FIG 5—Neoantergan-histamine and benadryl-histamine. Complete pA. assay. The whole assay of the two antagonists was done on the same animal: a separate piece of ileum was used for each concentration of antagonist. Before the addition of the antagonist the preparations were stabilized by a series of preliminary histamine injections: A-B 0.5 μ g histamine Tyrode; B-C 10 μ g histamine antagonistic solution; C-D 10 μ g histamine Tyrode; D maximum effects. Bath volume=18 c.c.

FIG 9—Neoantergan-histamine. After-effect of antagonist. Immediately after removal of the antagonistic drug from the bath there is a further increase of depression. H = double dose of histamine.



antagonist with that of another. Methods are described for making such comparisons in an efficient way and possible objections to a comparative method are discussed.

3 The activity of neoantergan, benadryl, pethidine, and atropine in antagonizing histamine and acetylcholine has been determined in terms of pA on the guinea-pig's ileum. All these antagonists act against both histamine and acetylcholine, though at widely differing concentrations and in a qualitatively different way, as shown, for instance, by the time taken for equilibrium conditions to be reached. By characterizing each drug-antagonist pair by four pA values a more complete picture can be obtained of activity as influenced by duration of action and by concentration.

I am indebted to Dr F Bergel, of Roche Products Ltd, for supplying Pethidine to Dr J S White, of Parke Davis & Co., for Benadryl, and to Dr R Wien, of May & Baker for Neoantergan. The special armatures for converting P O relays into fluid switches were designed and made by Mr B F Ballhatchet. The drawings are by Mr A Boura.

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SOME ACTIONS OF β -HYDROXY- $\alpha\beta$ -DIPHENYLETHYLAMINE

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Morphine being a pharmacologically active derivative of phenanthrene Dodds, Lawson, and Williams (1944a) sought a morphine analogue on the same lines as their discovery of stilboestrol—an intensely active analogue of another phenanthrene derivative, oestradiol. They found that diphenylethylamine and seventeen related compounds possessed in differing degrees some of the properties of morphine, these were depression of righting reflex in rats, elevation of blood sugar in rabbits, and hyperexcitability, pupil dilatation, and vomiting in cats. Some compounds of this series also possessed some analgesic properties when tested clinically. Of these the most promising was diphenylethanolamine or β -hydroxy- $\alpha\beta$ -diphenylethylamine, which may be considered to have the same structural relation to morphine as stilboestrol has to oestradiol. Later (Dodds *et al*, 1944b) it was shown to be effective only in cases where the pain was associated with pressure on nerves. The possible therapeutic uses of this substance, called M4 in their series, justified an investigation of its general pharmacological actions.

Tiffeneau, Levy, and Boyer (1928) found that diphenylethanolamine caused weakening of the beat and slowing of the isolated snail and frog heart, and also of the exposed heart of chloralosed dogs. The substance also caused relaxation of isolated intestine, transient fall of arterial pressure in chloralosed dogs, but gave a vasoconstriction of the perfused isolated frog leg. Hasama (1930) showed that the fall of blood pressure induced by the substance in urethanized rabbits was still obtained after vagotomy or atropine. The vessels of the isolated rabbit ear were dilated by diphenylethanolamine, even in the presence of atropine, since the dilator action could antagonize barium chloride vasoconstriction. Hasama suggested that the activities of the substance were the result of a direct toxic action on smooth muscle. Tainter (1933) and Dodds,

Lawson, and Williams (1944a) also noted the depressor action and the latter authors commented on the dilatation of the pupil and general hyperexcitability of unanaesthetized cats following intramuscular injection of the substance

In this paper is presented confirmation and extension of the previous work with an analysis of the site of action of the substance. The actions of β -hydroxy- $\alpha\beta$ -diphenylethylamine hydrochloride, which will be referred to as M4, were tested on the following mammalian tissues (a) small intestine, (b) cardiovascular system, (c) the pupil of the eye

METHODS

For studies on isolated intestines and heart, rabbits were killed by stunning. Pieces of small intestine were suspended in a bath of oxygenated Tyrode solution (formula in Bayliss 1924) at 37–38 C and pH 7.4, and the contractions of the longitudinal muscles recorded. The coronary arteries of the heart were perfused with oxygenated Ringer-Locke solution (formula in Bain 1938) at 37–38 C and pH 7.4, through a cannula in the aorta, movements of the right ventricle were recorded, the heart being steadied by pinning the apex. For perfusion of the hind quarters, rabbits and cats were used, the former were killed by stunning, the latter had been anaesthetized with chloralose (60–80 mg/kg. intravenously) for other experiments. A cannula was inserted in the lower aorta and the legs were perfused with Ringer Locke solution at 38 C and pH 7.4 by a Dale-Schuster perfusion pump, the inflow pressure being recorded with a mercury manometer.

Blood pressure changes were recorded in cats anaesthetized with chloralose (60 mg/kg intravenously in preliminary ether anaesthesia) or with nembutal (0.5 c.c. nembutal solution (Abbott)/kg intraperitoneally). Carotid arterial pressure was recorded by a mercury manometer. To assess the action of M4 on the vessels of different tissues the volumes of a hind paw of the opposite skinned hind leg, and of a 2-in. length of small intestine were recorded optically on photosensitive paper (Downman, Goggio, McSwiney and Young 1943). In these experiments the arterial pressure was also recorded optically on the same paper. The organs were enclosed in plethysmographs, the leg plethysmograph enclosing the skinned leg from upper thigh to ankle but the skin of the same paw and its venous drainage were left intact outside the plethysmograph.

For experiments on the pupils of cats slit-like pupils were produced in two ways. First, the animals were anaesthetized with chloralose (80 mg/kg intravenously) given in preliminary ether anaesthesia (McDowall 1925, Bain, Irving, and McSwiney, 1935). Secondly, under ether anaesthesia cats were decerebrated through a trephine opening in the cranium, the plane of section curving downwards and forwards from the upper edge of the inferior colliculi to the sphenoid eminence, leaving the hypothalamus and adjoining structures intact. The carotid arteries were temporarily occluded by clips and the vertebral arteries by digital pressure, but they were released as soon as possible. As the animal excreted its ether the pupils closed down to slits. If restoration of blood flow to the brain stem was delayed too long the pupils might not constrict. The oculomotor nerve could be exposed by tearing the dural covering over it on its way to the orbit.

Stock solutions of M4 were made by dissolving the hydrochloride in distilled water without heat, to give 2 or 5 per cent (w/v) solutions. Dilutions were made from the stock solution into the appropriate physiological salt solution. It should be noted that solutions of M4 are acid and simple neutralization throws the base out of solution. All doses are in terms of the hydrochloride. Injections into the cat were made into the superficial vein of the right foreleg.

RESULTS

(a) *Small intestine*

Isolated small intestine of rabbit was relaxed by M4 and the rhythmic movements diminished in amplitude. The lowest concentration found effective was 1 in 20,000. The loss of tone was rapid at first, but was followed by prompt recovery of tone and activity on washing (Fig. 1). M4 was active on the atropinized intestine and antagonized the spasm produced by acetylcholine, eserine, prostigmine, and barium chloride. Whereas M4 relaxed the gut, similar concentrations of morphine sulphate produced a slow increase of tonus.

Aqueous solutions of M4 being acid—e.g., pH 5.3 for 2 per cent (w/v) solution—it might be argued that the spasmolytic action represents no more than the action of an acid solution. Indeed there was a noticeable fall of pH, shown by adding phenol red to the Tyrode solution, after addition of an active quantity of M4. Advantage was taken of the buffering power of plasma. The pH of heparinized rabbit plasma was 7.40. After the addition of 1 volume of 2 per cent (w/v) aqueous solution of M4 to 4 volumes of plasma the pH of the mixture, measured with a glass electrode pH meter, was the same as that of the original plasma. The buffered M4 produced the same changes of intestinal activity as unbuffered M4, when each was added to the Tyrode bathing the intestine to the same final concentration of M4.

(b) *Cardiovascular system*

Isolated rabbit heart—0.1 to 0.2 c.c. of 2 per cent (w/v) solution of M4 injected quickly into the perfusion cannula produced a sharp decrease of amplitude, with not more than 15 per cent slowing of the beat. The heart recovered steadily in the next 5 minutes. Similar results were recorded when the same doses of M4 were diluted with 0.8 c.c. rabbit plasma before injection, whereas plasma alone usually produced a slight increase in the amplitude of beat.

Faradizing a vagus nerve supplying the isolated heart caused slowing and weakening of the beat. At the height of the M4 action vagal stimulation produced no or but slight slowing of the beat. It was noticed also that the amplitude of the beat might be a little increased during the stimulation.



FIG. 1—Action of hydroxy-diphenylethylamine upon longitudinal movements of rabbit duodenum suspended in Tyrode solution. M4 = addition of 2 per cent solution to give a final solution of 1/5000. W = wash. Time signal = 30 sec.

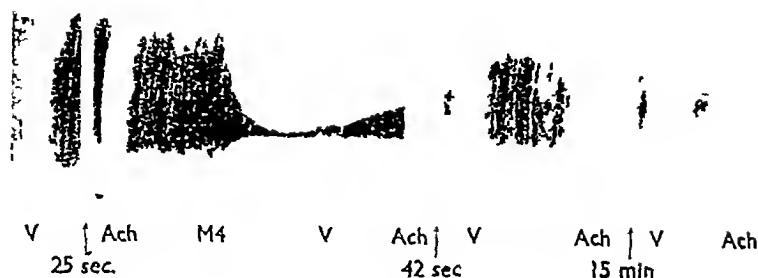


FIG 2—Action of hydroxy-diphenylethylamine on the isolated perfused rabbit heart. Tracing shows movements of right ventricle. M4= injection of 2 mg. hydroxy-diphenylethylamine HCl into aortic perfusion cannula, buffered by mixing 1 vol 2 per cent solution of M4 with 4 vol rabbit plasma. Ach= injection of 2 μ g acetylcholine in 0.2 cc Ringer-Locke solution. V=faradic stimulation of vagus nerve on the oesophagus. Time tracing=5 sec.

(Fig 2) The vagal inhibitory action returned as the heart itself recovered from the influence of the M4, but the full return of vagal response was not seen until some 5 minutes after the amplitude of the heart beat had fully recovered. The response to acetylcholine—e.g., 1 μ g in 0.1 cc Ringer Locke—was also reduced, and vagal and acetylcholine action was recovered at about the same speed. M4 action on the heart was not influenced by prior atropinization. Following a depressant dose of M4 the action of adrenaline was reduced but not abolished.

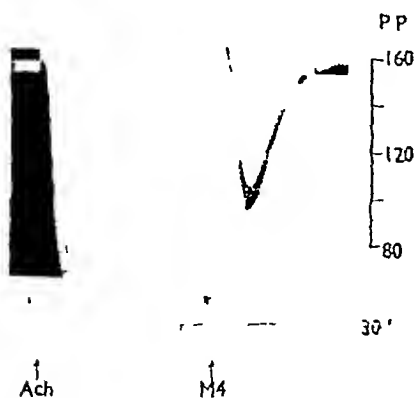


FIG 3—Vasodilator action of hydroxy-diphenylethylamine and of acetylcholine compared in cat hindquarters perfused Ringer-Locke solution, pH 7.5 containing 1:500,000 adrenaline. Ach=3 μ g acetylcholine. M4=2 mg. hydroxy-diphenylethylamine HCl. Time signal=30 sec. Perfusion pressure (PP) in mm Hg.

Perfused hind limb—In order to demonstrate the action of M4 or any vasodilator drug the tone of the vessels was raised by adding adrenaline to the perfusion fluid in a concentration of 2×10^{-6} to 3×10^{-7} . M4 now produced a transient dilatation of the limb vessels, shown by a fall of the perfusion pressure head (Fig 3). This dilator response, which was mimicked by 0.05 to 0.1 cc N/10 hydrochloric acid, cannot be attributed solely to the injection of an acid solution. It was obtained when the M4 was buffered adequately with plasma, as described above, although plasma injected alone produced a small rise of perfusion pressure.

The dilator action of M4 was seen when the limb vessels were constricted not only by adrenaline but also by posterior pituitary extract or barium chloride

Vascular responses in anaesthetized cats—Intravenous injection of M4 dissolved in 0.9 per cent sodium chloride solution produced a temporary fall of general arterial pressure (Fig. 4). With small doses arterial pressure recovered

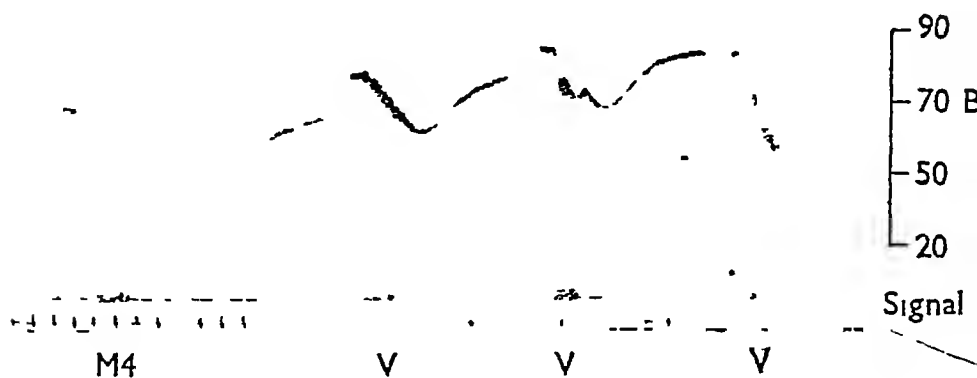


FIG. 4—Depressor action of hydroxy-diphenylethylamine 20 mg i.v., upon carotid arterial pressure of chloralosed cat (Signal for injection is 10 sec. late) V=faradic stimulation of peripheral end of cut right vagus nerve in neck. Time tracing=10 sec. B.P. calibration in mm. Hg.

quickly and was usually followed by a small but prolonged hypertension. In chloralosed cats 10 mg M4/kg produced about a 40-mm Hg fall of blood pressure with recovery in two minutes, while 50 mg/kg led to a 75-mm Hg fall of pressure with cardiac irregularity. Doses over 50 mg/kg caused cessation of breathing for periods up to 25 minutes.

Accompanying the fall of arterial pressure there was a rapid decrease of volume of the paw, skinned limb and intestine. At the same time the pulsations in these organs decreased in amplitude. As the arterial pressure recovered the organ volume and the amplitude of the pulsations returned. Similar changes were seen after bilateral vagotomy in the neck and inactivation of both carotid sinuses by tying the arterial trunks close to the sinus where they entered and left the structure. The shrinkage of the peripheral organs started at the same time in each of them and did not commence until the central arterial pressure had already fallen a little (Fig. 5).

The loss of vagus action in the presence of M4 could be shown in the intact animal by comparing the depressor response to faradizing the peripheral end of the cut right vagus nerve in the neck before and after injection of a dose of M4 which produced a prolonged action. The fall of pressure became smaller and the vagal inhibition of heart rate was much reduced, as in the isolated

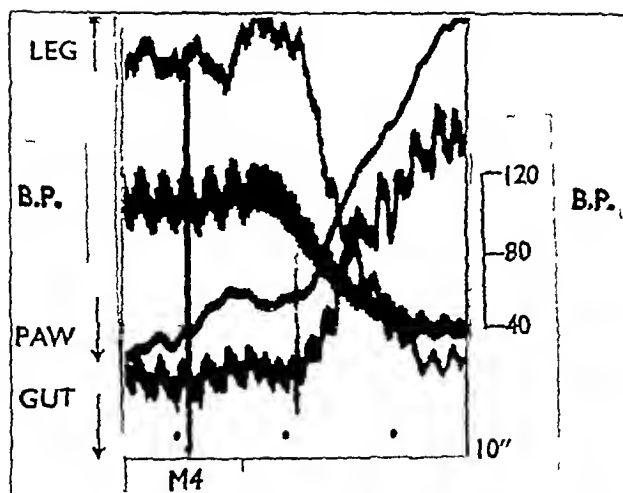


FIG 5—Optical record of carotid arterial pressure, with volumes of left hind paw skinned right hind leg, and segment of jejunum. Cat, nembutal i.p. At signal 30 mg hydroxy-diphenylethylamine i.v. Time marking=10 sec. B.P. calibration in mm Hg. Arrows show direction of vasodilatation.

heart, as blood pressure recovered so the vagus responses recovered, but the latter was not complete until some 1 to 5 minutes after the end of the M4 depressor response (Fig 4)

(c) Action on the pupil

In the chloralosed or decerebrated cat with slit-like pupils intravenous injection of M4, in doses of 12 mg/kg or more produced a rapid wide dilatation of the pupil with slow recovery. The whole effect lasted 10 to 30 minutes, and was not altered by cutting both cervical sympathetic chains in the neck. In cats with one oculomotor nerve severed it was possible to follow the dilator action of M4 upon the contralateral normal eye, and at the same time to test the ability of the oculomotor nerve to constrict the pupil by faradizing the peripheral end of the cut nerve. With the nerve severed the pupil was widely dilated, but constricted to a slit when the nerve was stimulated. At the height of the M4 action, as judged by the dilatation produced in the normally innervated eye, the constrictor effect of oculomotor stimulation was quite absent. As the pupil dilator action of M4 receded, so the ability of oculomotor nerve impulses to produce pupil constriction returned. Even when oculomotor action on the pupil was paralysed, stimulating the nerve still produced the usual rotation of the eyeball and enophthalmos. Stimulating the peripheral cut end of the cervical sympathetic chain in the neck produced a further slight dilatation

of the pupil when the latter was apparently fully dilated by M4, as well as movement of the nictitating membrane

M4 could antagonize the action of eserine. This was shown by cutting the oculomotor nerve on one side and then constricting the paralysed pupil by instilling into the conjunctival sac 0.65 mg eserine sulphate (1 Burroughs Wellcome "Tabloid"). Intravenous injection of a dose of M4, which produced full dilatation of the normal pupil, produced one-third dilatation of the eserinizied pupil.

Stereoisomers of hydroxy-diphenylethylamine

There being two asymmetric carbon atoms in the molecule two optically inactive stereoisomers are possible, the normal form (M4 itself) and an *iso* form. Each of these can be resolved into two optically active enantiomorphs. A comparison of the activities of these various isomers was attempted, using rabbit jejunum as the test object. More regular responses were produced if the gut was brought into high tone by suspending it in Tyrode solution to which eserine sulphate was added in a concentration of 1:4 million. The isomers were added to the gut bath as 2 per cent (w/v) solutions in water in an amount producing about half the maximum relaxation of the gut. The gut was exposed to each isomer for 5 minutes, then washed twice with Tyrode solution in the next 10 minutes. Even with this long exposure some pieces of gut did not reach their final length as the relaxation was rapid for the first two or three minutes and then proceeded very slowly.

Comparing the relaxation produced by similar concentrations of the isomers, 1:15,000, the activities of the isomers could be listed as *l-iso* > *d-normal* > *dl-iso* > *dl-normal* > *d-iso*. The *l-normal* form was not available. Equal relaxation was produced by 1:20,000 of the *l-iso* and 1:12,000 of the *d-iso*. It is difficult to assess the value of these results. Although there is a consistent difference between the *l-iso* and *d-iso* form, the activities of the first four isomers listed are very similar. Comparison of the vasodepressor action in the chloralosed or spinal cat was less successful. The responses to similar doses of the isomers were not always consistent, and might change during the experiment, but the results did not suggest any such difference of activity as shown by the gut.

DISCUSSION

Although hydroxy-diphenylethylamine (M4) apparently resembles morphine in having analgesic action, Dodds, Lawson, and Williams (1944) showed that these two substances probably act in different ways. Similarly, the previous results show that the general activities are also different. M4 is in general a depressor of smooth muscle action. Also it has an action which may for the moment be called "atropine-like."

It has been shown that the drug relaxes intestinal muscle and reduces the amplitude of the rhythmic contractions. This action is reversible and can be exerted against substances, such as barium chloride, which raise the tonus of the muscle. A similar spasmolytic action is produced in the blood vessels. These actions might all be explained by a general toxic action which directly reduces the power of the muscle to contract. Such a depressant action is seen in the heart, where M4 reduces considerably the amplitude but not the frequency of the beat. That this effect is not due to a parasympathomimetic action of the drug is shown by its occurrence in the atropinized heart. These findings agree with the reports of previous workers.

In view of the depressor action of M4 it could be argued that the fall of arterial pressure is a consequence of a peripheral vasodilatation, especially as M4 does have such an action in the isolated perfused limb. The simultaneous recording of paw, skinned limb, and intestine volumes shows however that in the whole animal the fall of arterial pressure is accompanied by a shrinkage of these organs. Since M4 is an active vasodilator in the isolated perfused limb, with maintained inflow, it seems that in the whole animal there is produced a passive vasoconstriction consequent upon the fall of arterial pressure. This is confirmed by the fall of pressure starting demonstrably earlier than the volume changes of skin, skeletal muscle, or intestine. The initial rapid fall of blood pressure on injecting M4 seems, therefore, to be due to a reduction of heart output because of the toxic action of the drug on the heart muscle. This toxic action is well shown on the isolated heart preparation.

The dilator action on the pupil in the unanaesthetized cat might be due to some central action in the brain stem or to a peripheral paralysis of the iris. That the neuromuscular mechanism of the iris is influenced directly is shown by the ineffectiveness of the oculomotor nerve stimulation in the presence of a dilator concentration of M4. It is probable, therefore, that a peripheral action would explain the pupil dilatation observed by Dodds and his colleagues. It is to be noted in the paper by Dodds, Lawson, and Williams that a dose of M4 which produced general hyperexcitability (20 mg/kg intramuscularly) was less than the pupil-dilating dose (50 mg/kg). A general toxic action on the whole neuromuscular mechanism does not seem to be an adequate explanation of the oculomotor paralysis. The dilator action of the sympathetic fibres was still present, arguing against an inability of the muscle fibres of the iris to react to nerve impulses, further, conduction in the oculomotor nerve was not impaired because it could still carry nerve impulses to the extraocular muscles, producing movement of the eyeball. The action of M4 on the pupil seems to be of an atropine-like nature, blocking conduction at the parasympathetic terminals. This action is, however, of short duration and is produced only in concentrations which have direct toxic actions on other smooth muscle.

The ability of M4 to block the action of parasympathetic nerve impulses

is seen also in its effect upon the vagal control of heart rate. This action has been observed only after doses of M4 sufficient to produce severe weakening of the beat, but does persist for a few minutes after the visible signs of this depression have worn off. It will be recalled that certain barbiturates produce a similar transient blocking of parasympathetic impulses and there is some evidence that the site of the block may be either in the ganglion or at the neuromuscular junction. Thus Koppanyi, Linegar, and Dille (1935) showed that some barbiturates, other than thiobarbiturates, produced a transient loss of vagal action and the evidence suggests that the site of action of the drug is mainly at the vagus ganglion. Stravinsky (1931) showed that amytal paralyses the submaxillary glands to chorda tympani stimulation, and since the acetylcholine response is also paralysed it appears that the drug acts on the nerve terminals or the secretory cells. Garry (1930) also has shown that amytal temporarily abolishes the action of the vagus nerve on the heart in cats and rabbits, the effect passing off much more rapidly in the rabbits, the action of acetylcholine on the frog heart was unchanged, but vagal activity was not tested at the same time in this animal. In the heart both vagal and acetylcholine activities are paralysed by M4 to the same degree, but whether this should be compared with the action of atropine is debatable, since M4 is so toxic to the heart muscle. It is possible that the effect represents no more than a general toxic action which takes a little more time to recede from the more fragile place where acetylcholine acts. The observations on the pupil, however, suggest that toxic and "atropine-like" action may be separate entities. It is curious that although M4 reduces the force of the heart beat very greatly it has relatively little action on the rate of firing of the pacemaker.

Although many of the actions of M4 may be due to non-specific depression of the tissues, there seems to be stimulation of some parts of the central nervous system. General bodily activity is increased (Dodds *et alia*, 1944), an action shown by diphenylethylamine (Tainter, Ludvena, Lackey, and Neurath, 1942). Some related compounds will even cause convulsions in cats. Some of the diphenylethylamine compounds also cause a rise of blood sugar (Dodds *et alia*, 1944), but the ability to cause hyperexcitability and raise the blood sugar are dissociated in M4, this substance producing hyperexcitability without hyperglycaemia. Clearly it would be of interest to know more of the cause of the hyperglycaemia provoked by other diphenylethylamine derivatives.

SUMMARY

A morphine analogue, β -hydroxy- α β -diphenylethylamine, has the following actions

- 1 It relaxes isolated intestine, even in the presence of acetylcholine, eserine, prostigmine, or barium chloride

2 The contraction of the isolated heart is reduced in amplitude, this action being unaffected by previous atropinization. Loss of response to vagal impulses and to acetylcholine is also produced.

3 It causes a fall of arterial pressure, with peripheral vasoconstriction. The latter is a passive effect. In the isolated perfused limb active dilatation is produced.

4 It produces pupil dilatation in chloralosed and decerebrated cats by an action on the oculomotor nerve terminals in the iris.

5 These results might be explained by a direct toxic action of the drug on the active tissue.

This investigation was undertaken after a suggestion by Prof. E. C. Dodds, M.V.O., F.R.S., whom I have to thank for original supplies of M4 and its isomers. The M4 was made by Boots Pure Drug Co., Ltd., to whom thanks are due. During the work much valuable assistance was given by Miss J. G. Emmett, of Boots Pure Drug Co., Ltd., working in this laboratory.

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THE THERAPEUTIC ACTION OF SOME KNOWN AMOEBICIDES IN RATS

BY

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Three main types of drug are used in the treatment of human amoebiasis firstly, drugs of the emetine type, e.g. emetine hydrochloride, emetine bismuth iodide, and auremetine, secondly, arsenical drugs, e.g. carbarsone and stovarsol, and thirdly, halogenated hydroxyquinolines, e.g. chiniofon, vioform, and diodoquin. Opinions differ concerning the respective merits of these three types, indeed the general opinion seems to be that no one type is entirely satisfactory in itself, and that the best clinical results are obtained by the judicious use of all three. As we now have a technique for evaluating the anti-amoebic properties of drugs, using experimentally infected rats (Jones, 1946), it was considered of interest to make a close comparison of the most commonly used of the above types. Such a comparison would also serve to supply standards against which any newly discovered anti-amoebic drug could be compared.

The drugs selected for the comparison were emetine hydrochloride, chiniofon, stovarsol, carbarsone, and diodoquin. They were compared when given as a single dose, and also when given according to a multiple-dose schedule.

EXPERIMENTAL WORK

Several experiments were carried out, each involving the use of 144 recently weaned rats weighing approximately 20–33 g. In each experiment the rats were separated into six groups of matched weights. They were then injected intracaecally, after laparotomy, with 0.25 c.c. of a suspension of *Entamoeba histolytica* in 5 per cent gastric mucin. The amoebae were cultivated in the enriched serum-buffered saline medium described previously (Jones, 1946).

Two different dosage schedules were employed. In the first a single dose was given 24 hours after the operation, and in the second doses were given 24, 30, 48, 54, and 72 hours after the operation. All doses were given orally, by means of a metal catheter. Emetine and chiniofon were given as solutions in water, and stovarsol, carbarsone, and diodoquin as finely dispersed suspensions made by ball mulling for several hours with a suitable dispersing agent (1 per cent Dispersol OG, I.C.I.).

In order to assess the therapeutic effect of the test drug the rats were killed six days after the operation. After this time the infection in the control group is maximal, and any therapeutic effect in treated groups is therefore most readily detectable. Careful post-mortem examinations were made of each rat in the experiment, and according to the degree of infection found a score was allocated. The six standard degrees of infection are shown in Fig. 1.

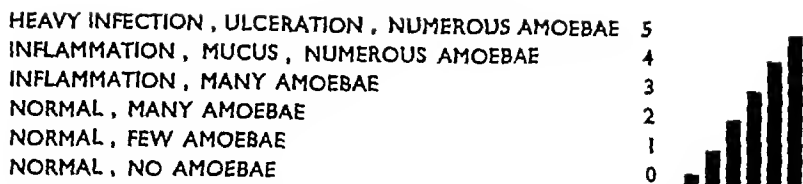


FIG 1—The degree of amoebic infection found in the caecum of experimentally infected rats

An average degree of infection (ADI) was calculated for each group by finding the average of the individual scores

The statistical significance of a treatment effect may be assessed according to the following formula

$$z = \frac{x - y}{\sqrt{\frac{\sigma^2 x}{m} + \frac{\sigma^2 y}{n}}}$$

where x and y are the ADIs for the control and treated groups respectively

σx and σy are the standard deviations for x and y respectively, and m and n are the numbers of rats in the control and treated group respectively

The values of σx and σy used in the above formula were read from a curve derived from the results of a large number of control and treated groups (Jones, 1946)

In this series of experiments most of the drugs, when given at the highest dose, showed a therapeutic effect of high statistical significance ($P < 0.01$). This value ($P < 0.01$) was therefore taken as the standard representing definite positive therapeutic effect (+). Therapeutic effects of significance $P = 0.05$ to $P = 0.01$ were regarded as indicating slight though definite therapeutic effect (\pm), whilst effects of $P > 0.05$ were regarded as indicating insignificant therapeutic effect (—)

The results of the comparisons are recorded in detail in Figs 2 and 3, and summarized in the Table

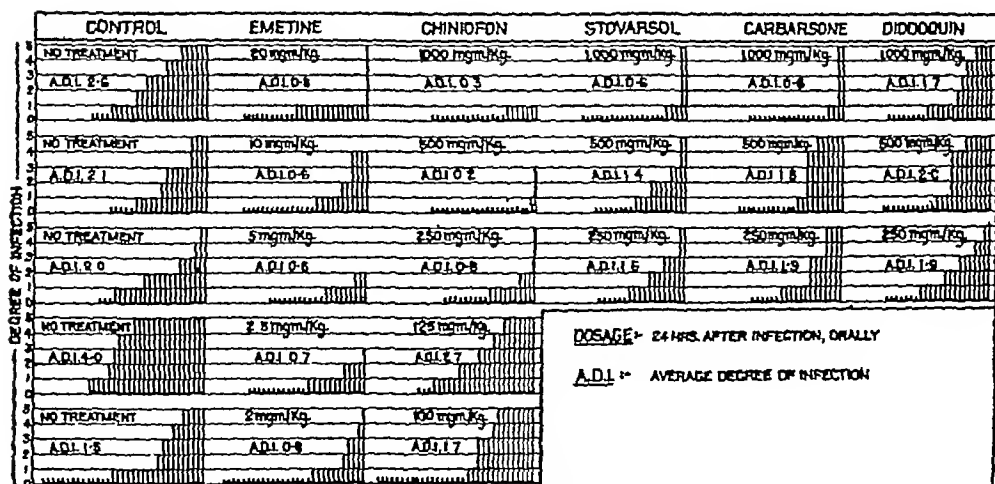


FIG 2—The effect of drugs on experimental amoebiasis in rats. Single-dose therapy

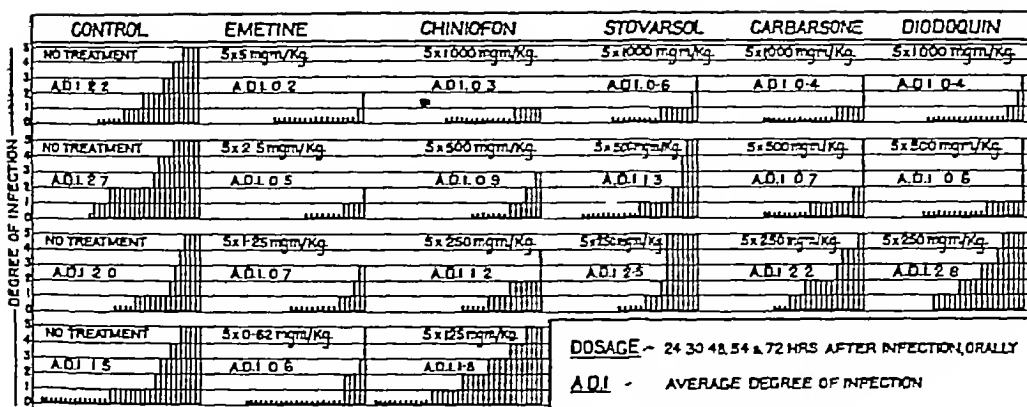


FIG 3—The effect of drugs on experimental amoebiasis in rats Multiple-dose therapy

TABLE
THE EFFECT OF DRUGS ON EXPERIMENTAL AMOEBIASIS IN RATS

Dose, mg./kg. orally 24 hr after operation		Significance of treatment				
		+ - P < 0.01 ± - P, 0.01-0.05 - - P > 0.05				
Emetine	Chiniofon, etc	Emetine	Chiniofon	Stovarsol	Carbarsone	Diodoquin
Single dose therapy	20	1000	+	+	+	+
	10	500	+	+	+	+
	5	250	+	±	—	—
	2.5	125	+	±	—	—
	2.0	100	—	—	—	—
orally 24, 30, 48, 54 and 72 hr after operation						
Multiple dose therapy	5	1000	+	+	+	+
	2.5	500	+	+	+	+
	1.25	250	±	—	—	—
	0.62	125	±	—	—	—

DISCUSSION

In comparing the results of our experiments in rats with the results obtained in human amoebiasis with the same drugs, we must consider certain important differences between the infections in the two species. Whereas in rats the infection is an acute one and is reasonably standardized, in human amoebiasis there is not only the acute disease but also a chronic phase of widely varying symptomatology. In the latter form of the disease we may have also to deal with the cystic form of the parasite, against which there is as yet no suitable means of testing drugs experimentally. If we compare our results with the results usually obtained in acute amoebic dysentery in man we find reasonable agreement. Thus emetine and chiniofon appear to be the most effective, with stovarsol,

carbarsone, and diodoquin definitely inferior. If, however, we consider the respective merits of these drugs in chronic amoebiasis, we find them at variance with our experimental results, for against this form of the disease, carbarsone, stovarsol, and diodoquin are undoubtedly of value. The minimal effective therapeutic dose of this type of compound in rat and man differs considerably if the comparison is made on a mg/kg basis. Thus a dose of 1,000 mg/kg, or 5 doses of 500 mg/kg, is required to produce an effect in rats, whereas the dosage used in humans, namely 4 gr twice daily for 10 days (Manson-Bahr, 1945), corresponds to a total dosage of only 80 mg/kg. The validity of such a comparison, however, is questionable, and it is perhaps more reasonable to consider the relationship of the minimal effective therapeutic dose to the toxic dose in the two species. If this is done, the relationship is found to be approximately the same.

It is of interest to note the behaviour of diodoquin in our tests. When it was given as a single dose, its therapeutic effect was barely significant, and compared unfavourably with carbarsone and stovarsol. When it was given repeatedly, however, its activity was better demonstrated. The fact that it is poorly absorbed was no doubt responsible for this difference. This compound has been introduced comparatively recently (Hummel, 1939). It does not appear to be much more effective than the other amoebicides (Morton, 1945).

The strain of *E. histolytica* used in this series of experiments (isolated in culture from material kindly supplied by Dr A R D Adams) was one which produced infections susceptible to treatment with emetine. Not all strains do so, as has been mentioned in a previous paper (Jones, 1946). It was decided to use an emetine-susceptible strain for comparison as there was no evidence that the other drugs showed similar differences in effectiveness against different strains. The comparison was accordingly carried out under conditions equally favourable to all the test drugs.

SUMMARY

A study has been made of the therapeutic action of emetine, chiniofon, stovarsol, carbarsone, and diodoquin against experimental amoebiasis in rats. Emetine and chiniofon appeared to have the widest range of activity. Stovarsol, carbarsone, and diodoquin were effective when given in large doses.

I thank the following: G H Davies, S R Smedley, D Todd, and W A. Whittaker for technical assistance, and Dr O L Davies for advice in the statistical assessment of the results.

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THE TECHNIQUE OF TESTING CHEMOTHERAPEUTIC ACTION ON *PLASMODIUM GALLINACEUM*

BY

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During the war *P. gallinaceum* and *P. lophurae* came into general use for testing the antimalarial action of new compounds. The purpose of the present work, carried out during 1943 and 1944, and withheld from publication owing to wartime restrictions, was to compare different methods of dosage in tests with *P. gallinaceum*, and to see how much the answer obtained depended upon the technique employed.

In all tests the compounds were judged by their ability to reduce parasitaemia to a low level, little or no account was taken of the power of drugs to sterilize the birds and thus to produce a radical cure of the infection.

METHODS

The general plan of the test was based on confidential information received from the American Board for the Co-ordination of Malaria Studies and from Mr D G Davey, of Imperial Chemical Industries, Ltd.

The strain of *P. gallinaceum* used was obtained from the Molteno Institute, Cambridge and was derived from the original strain introduced into Europe by Brumpt. It was maintained by blood passage in young chickens. The chickens were about 10 days old at the time of inoculation and weighed 60–80 g., they were infected by the intravenous injection of about 10^7 parasitized cells per chick. In a typical experiment the percentage of parasitized erythrocytes, resulting from this inoculum, was 1 per cent after 2 days, 6 per cent after 3 days, 30 per cent after 4 days, and 50–90 per cent after 5 days at which time chickens often died, if they survived, the parasites in the blood became less numerous but many died with exoerythrocytic forms in the brain two weeks after inoculation. The drugs were given by mouth with a syringe and blunt needle. Since most drugs supplied for testing are very limited in amount it was considered that the administration of drugs in the diet (as described by Marshall *et al.* 1942) would not be practicable. The blood of the infected chicks was examined on the 3rd and 5th days and the percentage of parasitized erythrocytes on the 5th day was determined.

To test for the prophylactic activity of drugs, the heads and thoraces of batches of mosquitoes known to contain sporozoites in their salivary glands were ground in saline and the resultant suspension centrifuged lightly to throw down the chitinous parts of the insects. The supernatant was drawn off and made up in a mixture of heparinized chick

plasma and saline, containing at least 50 per cent (v/v) plasma, so that 0.2 c.c. contained the equivalent of one infected mosquito. This suspension was used to infect chicks by intravenous inoculation of 0.2 c.c. per chick. Chicks so infected usually exhibited parasites in the peripheral blood 5-7 days after inoculation, and died 6-10 days after infection as a result of massive infection of the endothelial cells of the cerebral capillaries with exoerythrocytic forms. The drugs were administered in the manner described above, dosage being started 2 hours before infection. In both types of experiment the geometrical mean of the responses of the individual birds in a group was taken, this was compared with the geometrical mean of the responses of the group of untreated control chickens.

The following salts of quinine, mepacrine (atabrine), and pamaquin (plasmoquin) were used: quinine bisulphate containing 59 per cent anhydrous quinine, mepacrine methanesulphonate (quinacrine soluble, May & Baker) containing 65 per cent of mepacrine base (3rd ADD B.P. 1932, p. 15) and the methylene bis-hydroxynaphthoate of 6-methoxy-8- β -diethylamino- α -methylbutyl-aminoquinoline (pamaquin) containing 45 per cent of base (4th ADD B.P. 1932, p. 24). All amounts of the drugs quoted refer to these salts. Sulphadiazine powder was used as the pure substance.

Measurement of the blood concentration of the drugs

An attempt was made to measure the levels of quinine, mepacrine, and sulphadiazine in the blood after the different dose schedules employed. In order to facilitate the taking of blood samples, larger chickens were used in these experiments than in the therapeutic experiments.

Quinine—The blood level of quinine at varying periods of time after dosing was measured by a modification of the method of Kelsey and Geiling (1942), kindly devised by Prof. C. Rimington. The estimations were carried out on 0.5 c.c. blood, drawn from the heart, of 3-4 months old chickens weighing about 800-1,000 g. A series of blood samples was taken from each chicken. The oxalated blood sample was pipetted into 3.5 c.c. distilled water and the blood proteins were digested by the addition of 1 c.c. 10 per cent sodium hydroxide and heating in a water bath for 15 min. The samples were then extracted with 25 c.c. of sodium-dried ether containing 5 per cent (v/v) petrol in a 50-c.c. separating-funnel. The ether extract was washed twice with 10 c.c. *N*/10 sodium hydroxide and the quinine extracted with 4 c.c. *N*/10 aqueous sulphuric acid in three successive volumes of 15 c.c., 15 c.c., and 1 c.c. of acid. The acid extract was then made up to 5 c.c. by the further addition of sulphuric acid and its fluorescence was measured in the Rimington fluorescence comparator (Rimington, 1943). The amount of quinine present in the extracts was ascertained by comparison with a standard curve obtained by measuring the fluorescence observed when known amounts of quinine were added to blood and extracted as above.

Mepacrine—For the estimations of mepacrine the appropriate doses were given to 800-1,000 g. chickens and blood was withdrawn from the heart. Repeated bleedings of the same chickens sometimes led to false readings of the drug level, consequently several birds were placed on the same dose schedule and no bird was bled more than three times. Coagulation of the blood was prevented by potassium oxalate, if the sample had to wait more than 24 hours before examination it was stored at -12° C. The estimations were kindly carried out for us by Major J. Reid of the Royal Army Medical College, he used a modification of Masen's method (1943).

Sulphadiazine—Appropriate doses of sulphadiazine were given to 28-day-old chicks. 0.02 c.c. blood was taken from the leg at intervals, and the concentration of sulphadiazine therein was measured by a modification of the method of Marshall and Cutting (1938). The volume was measured in special micro-pipettes and washed out into 2.5 c.c. of acid.

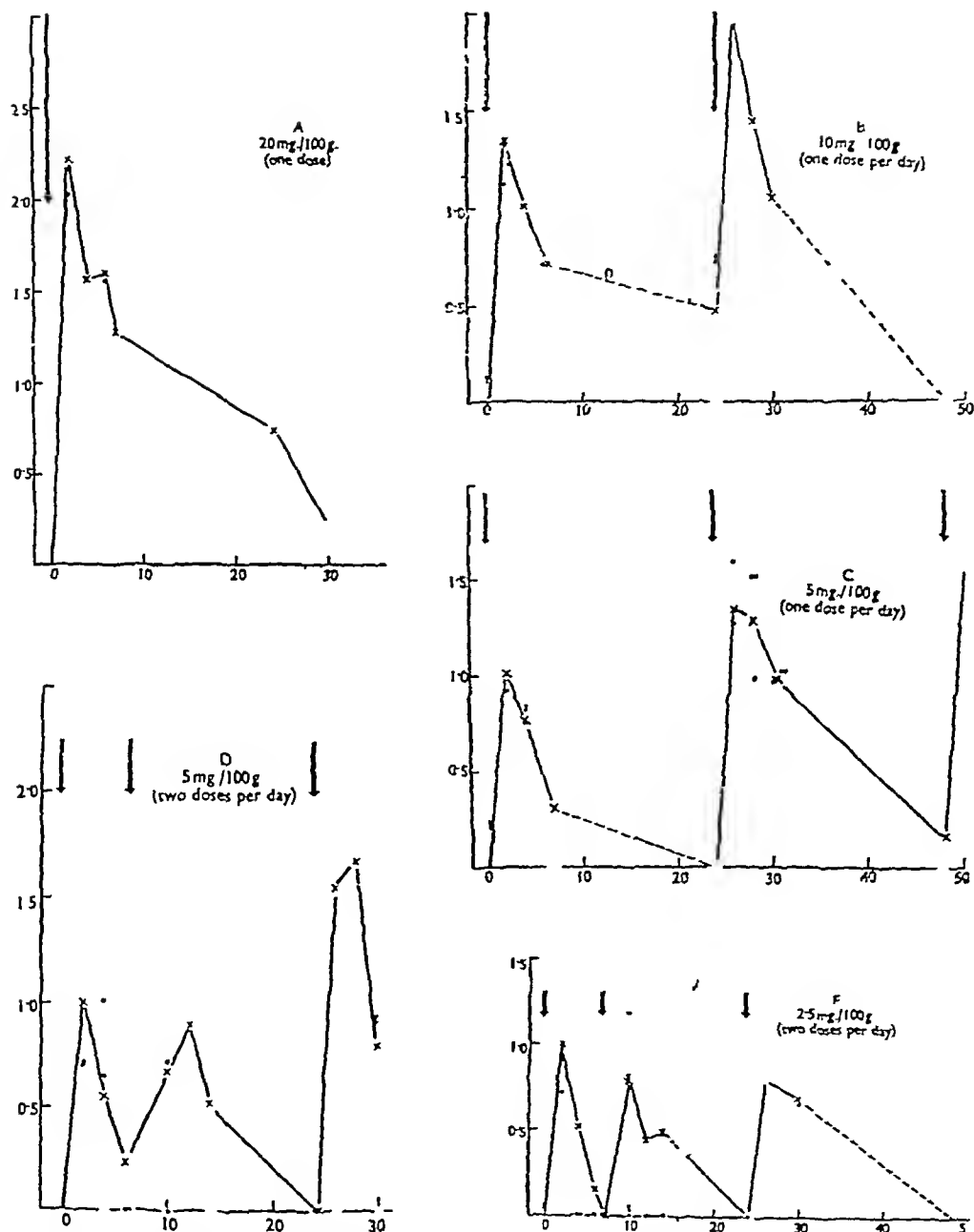


FIG 1—Blood concentrations of quinine in chicks after oral administration of different dose schedules of quinine bisulphate. Ordinates blood concentrations in mg. per litre. Abscissae hours after dosage. • = levels in individual birds x—x = average levels. Arrows indicate dosage.

saline (0.85 per cent NaCl in *N*/50 HCl). The reagents, 0.1 c.c. 0.3 per cent sodium nitrite, 0.1 c.c. 1.5 per cent ammonium sulphamate, and 0.1 c.c. 0.1 per cent naphthyl ethylenediamine dihydrochloride, were then added to 2.5 c.c. of the supernatant, and the intensity of the fully developed colour was measured on a Spekker absorptiometer combined with a spot galvanometer.

EXPERIMENTS

TROPHOZOITE-INDUCED INFECTIONS

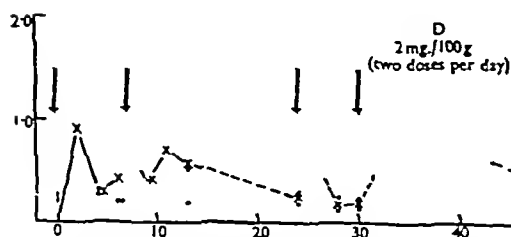
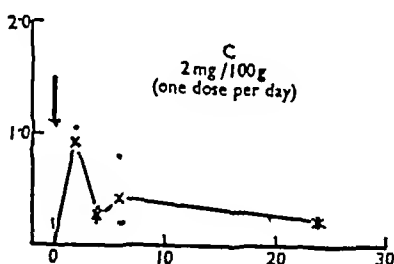
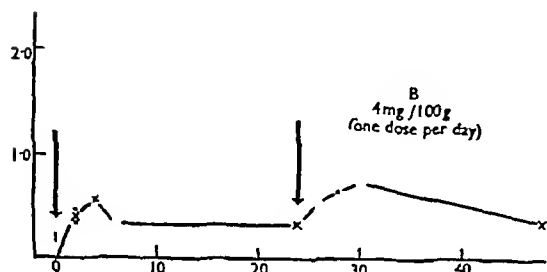
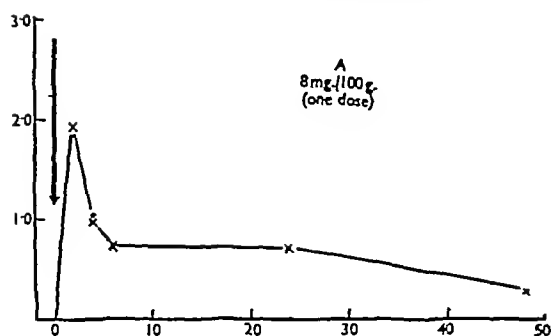
Effect of concentration or dispersion of the dose

The first investigations were made to determine the effect of concentrating the whole amount of antimalarial compound (quinine, mepacrine, pamaquin, or sulphadiazine) into a single administration or of dispersing it over several days in a series of smaller doses. The total amount administered remained the same, an amount being employed which was about the minimum effective level when given according to the most effective regime. A study was made of the results of the different dose schedules on (A) the resultant blood-concentration, (B) the therapeutic response.

A—Blood concentrations

Quinine—Fig. 1 shows the mean curve for the group of birds receiving quinine, it was obtained by taking the average of the blood concentrations at each particular time. Considerable individual variation in the levels was observed in different chickens, particularly in the series 5 mg. twice daily for 2 days and 2.5 mg. twice daily for 2 days. The mean curve was only an approximate indication of what might happen in any special instance. On the whole the levels were comparable with those found by Kelsey *et al.* (1943). The absorption of quinine was rapid, the peak in the blood concentration occurring at approximately 2 hours, but the compound soon disappeared from the blood again, so that the period of antimalarial action was presumably brief in most cases. The level attained in the blood was not directly proportional to the size of the dose, since a dose of 2.5 mg. produced a peak mean blood concentration of 1.0 mg. per litre (Fig. 1E), while a dose of 20 mg. (8 times as great) produced a peak of 2.1 mg., only twice as high (Fig. 1A).

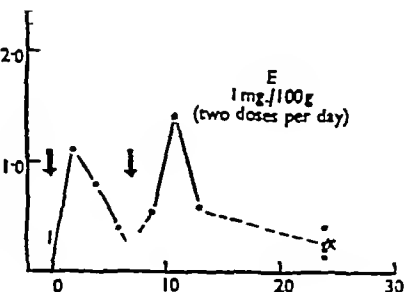
Mepacrine—The blood concentrations of mepacrine are shown in Fig. 2, the line being drawn through the mean concentrations at the different periods of sampling. The variations between the different birds were greater than those between the different dose schedules so that from this small number of birds no reliable conclusion could be drawn. On the whole, it appeared that with mepacrine there was a peak in the blood concentration occurring about two hours after the dose, followed by a prolonged plateau at a lower level. This is well exemplified in the curve obtained after the administration of 8 mg. per 100 g. (Fig. 2A). Marshall and Dearborn (1946a) found that in the treatment of *P. lophurae* infections in ducks by mepacrine the therapeutic response was



proportional to the dosage rather than to the plasma concentration of the compound. Our own limited figures on blood concentrations and on therapeutic response (Table I) are in agreement with their finding.

Sulphadiazine—The average blood concentrations obtained when sulphadiazine was given are shown in Fig 3. With one dose per day, even with the largest amount, the drug remained in the blood for less than 24 hours. The mean peak level varied with the dose, but not in direct proportion. After a dose of 25 mg the peak was 135 mg per litre, after 200 mg (8 times the dose) the peak was 254 mg. (Fig 3A and E). The effect of subdividing the doses was to prolong the periods in which appreciable amounts of drug were present in the blood and to make them more continuous. There was little evidence of an accumulation of sulphadiazine in the blood (Fig. 3E).

FIG 2.—Blood concentrations of mepacrine in chicks after oral administration of different dose schedules of mepacrine methanesulphonate. Ordinates blood concentrations in mg. per litre. Abscissae hours after dosage. • = levels in individual birds x—x = average levels. Arrows indicate dosage.



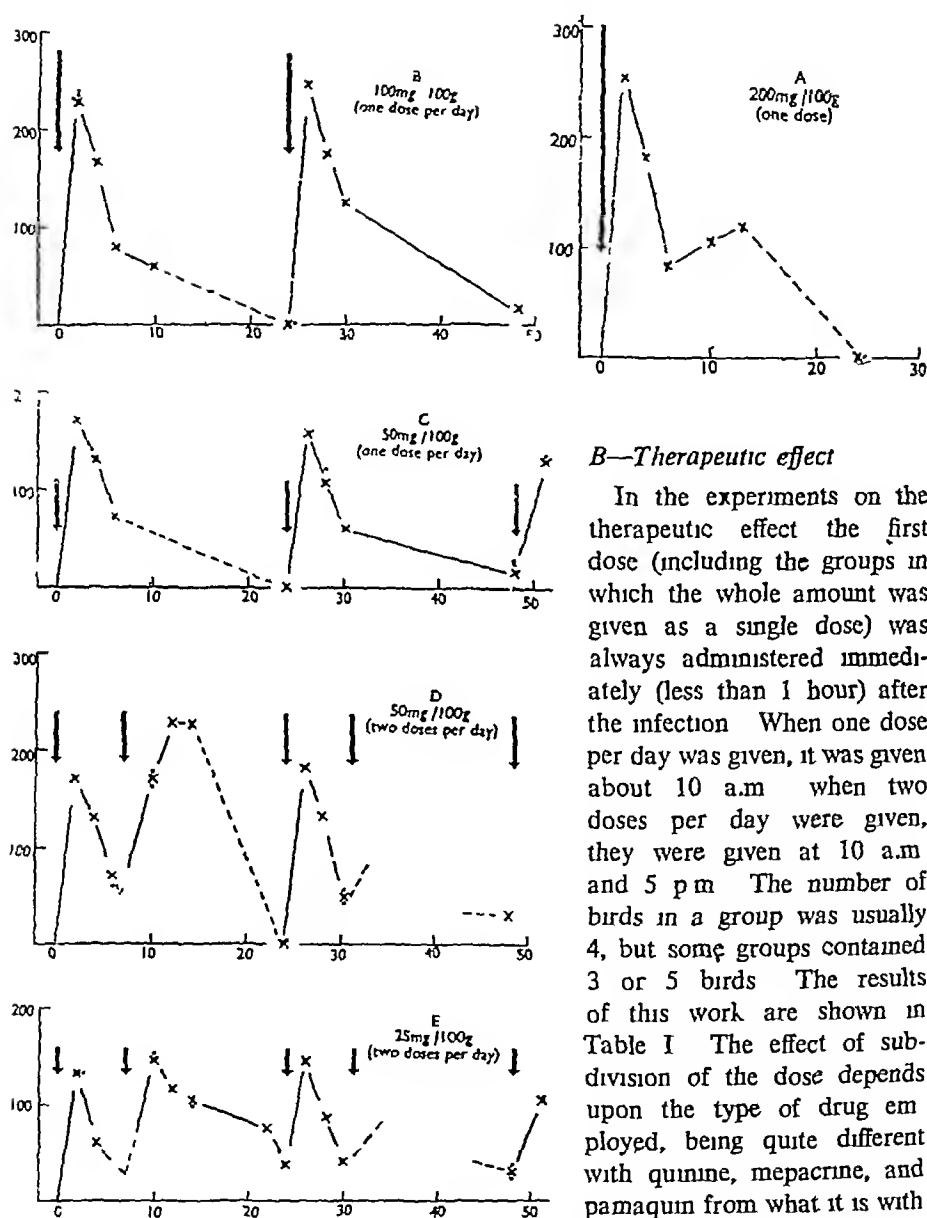


FIG 3—Blood concentrations of sulphadiazine in chicks after oral administration of different dose schedules of sulphadiazine powder. Ordinates blood concentrations in mg per litre. Abscissae hours after dosage. \times = levels in individual birds $-\cdot-$ = average levels. Arrows indicate dosage.

B—Therapeutic effect

In the experiments on the therapeutic effect the first dose (including the groups in which the whole amount was given as a single dose) was always administered immediately (less than 1 hour) after the infection. When one dose per day was given, it was given about 10 a.m. when two doses per day were given, they were given at 10 a.m. and 5 p.m. The number of birds in a group was usually 4, but some groups contained 3 or 5 birds. The results of this work are shown in Table I. The effect of subdivision of the dose depends upon the type of drug employed, being quite different with quinine, mepacrine, and pamaquin from what it is with sulphadiazine. With the former three compounds, the more the dose is concentrated the greater the response, and conversely. The drugs are

TABLE I
THE EFFECT OF DIFFERENT DOSE SCHEDULES OF STANDARD DRUGS ON THE RESPONSE OF
TROPHOZOITE-INDUCED INFECTIONS

Drug	Total dose mg./100 g	Percentage of cells parasitized on 5th day after different dose schedules					
		Total dose on 1st day	1/2 dose once daily 2 days	1/4 dose twice daily 2 days	1/4 dose once daily 4 days	1/8 dose twice daily 4 days	Untreated controls
Quinine	20	4.1	0.52	0.59	4.7	21.6	39.3
		1.95	1.18	1.20	24.4	17.2	53
		—	—	4.18	21.9	—	58.2
Mepacrine	8	0.61	1.30	52.3	48.9	47.9	59.5†
		0.1	0.1	1.8	3.7	—	38.5
	10	0.1	0.1	0.1	1.2	3.1	65.7
Pamaquin	0.4	—	—	5.24*	56.7	—	54.8
	0.4	0.1	—	2.9	70.9	54.7	58.3
	0.6	1.06	—	—	3.09	9.85	44
		0.1	0.1	1.35	17.4	2.51	63.9
	1	0.1	0.1	0.1	0.1	1.2	41.4
Sulphadiazine	200	37	28.8	38.7	12.6	0.12	39.5

* Poor test. Only 1 chick remained alive.

† 6th day count.

most effective if given in a single large dose or if the dose is subdivided only into two, as the subdivisions get more numerous, less antimalarial effect is shown. Apparently the action of these drugs upon the parasites is rapid, a short interval of time being sufficient for it to take place, but on the other hand it is strongly effective only if a certain level of blood concentration is reached—e.g., with quinine, a level of over 1 μ g per c.c., this critical level of concentration is presumably not reached when the dose of drug administered falls below a certain quantity, even if the dose is repeated twice daily for four days. With sulphadiazine on the other hand, the greatest antimalarial effect is obtained when the administration is spread over the whole four days, subdividing the amount into 8 small doses. The administration of the drug in 1–4 doses concentrated into the first two days is non-effective. Even the slight modification of giving a single dose on each of four days, instead of two smaller doses on each of these days, greatly diminishes the antimalarial effect. This class of compound presumably exerts a slower action upon the parasites than quinine, mepacrine, or pamaquin do, and the duration of action is more important than the concentration over any particular short period.

Effect of time of starting the treatment

It has been reported from America that some workers begin treatment several hours before infecting the birds, others wait until after the inoculation to begin treatment. The latter procedure is the more convenient in practice as the whole

batch of chicks can be inoculated without the necessity of identifying each individual in the process, moreover, birds in which the inoculation has been unsatisfactory in any way can easily be discarded and replaced by new ones. An investigation was made in order to discover whether this difference of procedure affected the therapeutic response observed, and the results are shown in Table II. As will be seen, there was no significant difference in the response whether the first dose was given immediately after inoculation, or 5 hours before inoculation. Accordingly, in our routine testing of drugs the first dose is always given almost immediately (less than 1 hour) after inoculation.

TABLE II

THE EFFECT OF THE TIME OF STARTING DOSAGE ON THE RESPONSE OF TROPHOZOITE INDUCED INFECTIONS

Drug	Total dose mg/100 g	Dose given twice daily for 14 days	Percentage of cells parasitized on 5th day when dosage started		Controls
			immediately after infection	5 hours before infection	
Quinine	20	2.5 mg	10.7 3.02 29	19 14.8 24.9	38.5 41.4 61.1
	24	3 mg	6	7.3	44
Mepacrine	10	1.25 mg	28.3 0.96 9.01	21.5 1.16 5.52	41.4 44 61.1
Pamaquin	0.4	0.05 mg	45.9	39.2	38.5
	0.6	0.075 mg	21.1 7.94	28.1 12	44 63.9
	1	0.125 mg	1.2	4.59	41.4
Sulphadiazine	200	25 mg	0.42	0.8*	39.5

Each of the groups of treated chicks contained 4 birds, the groups of controls usually contained 6-10 birds.

* Started 2 hours before infection in this case as sulphadiazine reaches its peak in the blood 2 hours after oral administration.

A further study of the effect of delay in commencing treatment was made by giving the same total amount of mepacrine either all on the last (3rd) day after inoculation or spread out over the previous days. This is a reverse of the dose schedules described in Table I. The results are shown in Table III. The best therapeutic responses were obtained when the drug is concentrated on to the last two days of the treatment. Probably in this experiment the effect of concentrating the dosage was more important than the time of starting it. (On this occasion mepacrine seems to have been less effective than in the experiments of Table I.)

TABLE III
THE EFFECT OF DELAY IN COMMENCING TREATMENT WITH MEPACRINE

Drug	Schedule	% cells parasitized on 5th day	Control
Mepacrine	6 mg twice daily on the 4th day	2.62	65.7
	3 mg " " " " 3rd and 4th days	0.56	
	2 mg. " " " " 2nd, 3rd, and 4th days	18.7	
	1.5 mg " " " " 1st, 2nd, 3rd, and 4th days	35.2	

In each group the total dose was 12 mg per 100 g
Each group contained 4 chicks.

SPOROZOITE-INDUCED INFECTIONS

Similar investigations about the effect of concentrating or dispersing the treatment were made with sporozoite-induced infections—i.e., the prophylactic action of the compounds upon the pre-endoerythrocytic forms of the parasites was studied instead of the therapeutic action upon the endoerythrocytic ones (trophozoites, schizonts, etc). At the time of conducting this work the only compounds known to have a prophylactic action were the sulphonamides, and accordingly the investigations were made using sulphadiazine. Two types of experiment were made. In the first, a given amount of sulphadiazine (200 mg per 100 g) was either concentrated at the beginning of the infection or dispersed over the first four days, in the second, treatment was more intense (total 800 mg per 100 g) and it was given either in the first two days or on the 3rd and 4th days after infection. The results of both these investigations are shown in Table IV.

In the first type of experiment the data show that the more the treatment was spread out over the first four days the more effective it was, treatment restricted to the first day had little or no effect, even if the total dosage was doubled (i.e., total dose 400 mg). These results with pre-endoerythrocytic stages of the parasite agreed with those obtained concerning the action of sulphadiazine upon the endoerythrocytic forms, they support the view that the antimalarial action of sulphadiazine is of such a type that duration of exposure is more important than intensity.

The second part of these experiments concerns the sensitivity of the different stages of the parasite to sulphadiazine. Treatment restricted to the first day had little or no action, apparently the sporozoites in their original form or in their early development after inoculation were not much affected by the compound during a single day. Treatment given on the 3rd and 4th days was slightly more effective in delaying the development of patent infection than that given on the 1st and 2nd days, but much of this effect was due to the postponement of treatment, and if the interval is measured between the onset of the patent infection and the end of treatment, there was little real difference between the degree of parasitaemia observed in the two groups. However ultimate survival was much

TABLE IV

EFFECT OF DIFFERENT DOSE SCHEDULES AND OF TIME OF DOSAGE OF SULPHADIAZINE ON SPOROZOITE INFECTIONS

	Total dose mg./100 g	Individual doses per 100 g	Days of treatment	Percentage of cells parasitized on day						Remarks
				8	9	10	11	12	13	
First part of experiment	200	*200 mg once daily	1st		24.6 2 D EEF+		23 1 D EEF+			
	200	100 mg once daily	1st, 2nd		1.2	2 D EEF+	49.3 2 D			
	200	50 mg once daily	1st, 2nd, 3rd, 4th		Less than 0.1		1.6		14.1 2 D EEF+	2 chicks died on 17th day
	200	*50 mg twice daily	1st, 2nd		Less than 0.1		0.66		14.9	3 chicks died on 15th day EEF+
	200	*25 mg. twice daily	1st, 2nd, 3rd, 4th		Less than 0.1		Less than 0.1			On 15th day 51.2. 1 D EEF+ 1 chick died on 16th day 1 chick sur- vived
	Control	*—	—		45.5 2 D		1 D			
	200	*200 mg	1st	10.2	1 D	48 2 D EEF+	1 D			
	200	25 mg twice daily	1st, 2nd, 3rd, 4th	Less than 0.1		Less than 0.1	0.4			2 chicks failed to show parasitaemia. 2 chicks died on 17th day EEF+
	Control	—	—	8.03 1 D	1 D EEF+	32.2	2 D			
	800	200 mg twice daily	1st, 2nd	Less than 0.1		1.2		6.9	2 D	2 chicks died on 14th day EEF+
Second part	400	200 mg twice daily	1st	4.3	1 D EEF+	11.6	1 D	1 D		1 chick died on 14th day
	Control	*—	—		45.5 2 D		1 D			
	800	*200 mg. twice daily	1st, 2nd	Less than 0.1		3.1	1 D EEF+		42.5 2 D	
	800	200 mg twice daily	3rd, 4th	Less than 0.1		Less than 0.1			24.3	1 chick died on 17th day EEF+ 3 chicks survived
	Control	—	—	8.03 1 D	1 D EEF+	32.2	2 D			

The inoculum used in these experiments contained the approximate equivalent of 1 infected mosquito per chick. The groups marked with an asterisk contained 3 chicks, the others contained 4. The '1st day' dose was given 2 hours before infection with sporozoites. D, Chick died EEF+ Exoerythrocytic forms found in the brain post mortem

better in the group treated on the 3rd and 4th days. It is noteworthy that (with the possible exception of 2 chicks) sulphadiazine in these doses failed to sterilize any of the birds. It only delayed the multiplication of the parasites for a shorter or longer period. In this respect its action upon pre-endoerythrocytic forms was indistinguishable from that upon endoerythrocytic forms. The results thus suggest that the antimalarial action of sulphadiazine is exerted both on pre-endoerythrocytic forms of *P. gallinaceum* (cryptozoites, etc.) and on endoerythrocytic forms (trophozoites and schizonts), that this action tends to be plasmodiostatic rather than plasmodiocidal, and that its effectiveness depends upon the duration of exposure rather than upon the intensity.

DISCUSSION

It has been shown that under the experimental conditions described the maximum effect of a given small quantity of quinine, mepacrine, or pamaquin upon the trophozoites is obtained when the treatment is concentrated into one or two days. The greatest effect of a given quantity of sulphadiazine upon either trophozoites or upon pre-endoerythrocytic forms is obtained when the treatment is spread out over the whole period of four days. With the former drugs, intensity of action seems more important than duration, with sulphadiazine, duration is more important than intensity. In the treatment of trophozoite-induced infections there is no significant difference in the response observed whether the first dose is given 5 hours before inoculation or immediately after it. The action of sulphadiazine is exerted both on pre-endoerythrocytic forms of *P. gallinaceum* and on endoerythrocytic ones.

A comparison has been made by Marshall and Dearborn (1946b) of the single-daily-dose and of the drug-diet methods of treatment in bird-malaria (*P. lophurae* in ducks) in relation to the therapeutic activity of numerous compounds, as found above, they showed that the relative activities of the different compounds are considerably affected by the concentration or dispersion of the dosage.

These results may now be considered in relation to the devising of routine tests of the antimalarial action of new compounds. If the compound were given in a single dose (as is customary in experiments with mouse trypanosomiasis) the action of quinine, etc., would be manifested while that of sulphadiazine would probably be missed. The maximum dose which can be tolerated depends upon the toxicity of the compound, and this is usually such that a series of doses spread over four days is much less injurious than a large dose on a single occasion. Accordingly the usually accepted regime of treatment spread over four days has much to recommend it since (1) it provides sufficient duration for the slower acting compounds such as sulphadiazine, (2) it minimizes toxicity and allows larger amounts of the compound to be given, and (3) it is not unduly laborious to administer. No experiments have been made in this work using schedules extending over more than four days, as a routine measure such schedules would appear

to be more wasteful of labour and of material (which is often scarce) while offering insufficient compensating advantage. The routine tests carried out in this laboratory are therefore based on treatment lasting four days. The general procedure has been described above in the section upon methods. For infections induced by trophozoites the response is read as the percentage of parasitized erythrocytes on the fifth day, and the geometrical mean for the group of birds is compared with that for the group of untreated controls. If a compound is found to be active in the maximum tolerated dose, the dose is reduced in subsequent tests until the lowest dose is found which reduces the percentage of parasitized cells to about one or two. This is considered to be the approximate minimum effective (therapeutic) dose. For infections induced by sporozoites, the chief criteria of the activity of a drug are complete suppression of infection or a delay in the appearance of parasites in the peripheral circulation and prolongation of the life of the chicken or recovery from the infection. For quantitative determination of the activity, measurement is made of the minimum effective (prophylactic) dose which prevents the percentage of parasitized cells being greater than 1-2 on the 7th to 9th day when the infection has reached a high level in the controls. No compound tested to date in this laboratory has been sufficiently effective for its activity to be expressed as the minimum dose which completely prevents infection. The antimalarial activity, therapeutic or prophylactic, as expressed by these minimum effective doses, is compared with the toxicity as determined by the maximum tolerated dose for mice, when administered orally twice daily for four days. Since the drugs are designed for use in man, the toxicity for mammals is more significant than that for birds, even though the tests for antimalarial potency are carried out in chickens.

SUMMARY

The methods used in the authors' laboratory for testing the antimalarial action of drugs upon infections of *P. gallinaceum* induced by trophozoites and sporozoites are described.

A given amount of quinine, mepacrine, and pamaquin exerts the maximum effect on trophozoite-induced infections of *P. gallinaceum* if it is concentrated into the first day or first two days of treatment, with these compounds intensity of action is more important than duration. A given amount of sulphadiazine produces the maximum effect upon trophozoite- or sporozoite-induced infections if it is dispersed over all the four days of treatment, with this compound duration of action is more important than intensity. The action of sulphadiazine is exerted both on the pre-endoerythrocytic forms of *P. gallinaceum* (cryptozoites, etc) and on the endoerythrocytic forms (trophozoites, etc).

In the test on trophozoite-induced infections there is no significant difference in the response whether the first dose is given immediately after the inoculation or 5 hours before it.

The blood concentration curves of quinine, mepacrine, and sulphadiazine on the different dose schedules were determined. Increasing the dose eight times increases the peak concentration in the blood only about twice.

Grateful acknowledgments are due to Miss Ann Bishop and Sir Rickard Christophers F.R.S., for provision of strains of parasites and mosquitoes and for help in the technique of breeding of mosquitoes, to the American Board for the Co ordination of Malaria Studies, and to Mr D G Davey for much confidential information about antimalarial tests to Major J Reid and Prof C Rimington for help in the chemical estimations of drugs in the blood, and to Miss R I Berson and Miss V D Markham for technical assistance.

The drugs used were kindly supplied by May & Baker Ltd Dagenham, and Imperial Chemical Industries, Ltd, Manchester.

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THE TOXICITY OF ALKYL FLUOROPHOSPHONATES IN MAN AND ANIMALS

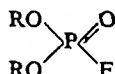
BY

B A KILBY AND M. KILBY

From the Physiological Laboratory, Cambridge

(Received March 13 1947)

In May, 1940, we prepared the dimethyl and diethyl esters of fluorophosphonic acid and tested their effects on animals as lethal inhalants. When, about a year later, McCombie and Saunders prepared the *isopropyl* ester, we found it had an even more powerful effect than the dimethyl and diethyl compounds.



R = CH₃, C₂H₅ or (CH₃)₂CH

dialkyl fluorophosphonate

The present paper deals with this early and first systematic study of the toxicity of the fluorophosphonates which are now widely used for physiological experiments and clinical trials. When the toxic action was discovered it was not possible for us to publish the results, which were, however, circulated as a report to the Ministry of Supply (Adrian, Kilby, and Kilby, 1940).

The preparation of the fluorophosphonates and this study of their toxic properties was prompted by an observation of Lange and Krueger (1932). They had prepared the dimethyl and diethyl compounds and had stated at the end of their communication that inhalation for a few minutes might lead to difficulty in breathing, to disturbance of vision, hypersensitivity to light, and even to loss of consciousness.

METHODS

The dimethyl and diethyl fluorophosphonates used in these experiments were prepared by the method described by Lange and Krueger, and the *isopropyl* ester was kindly supplied by Dr H McCombie, Dr B C Saunders and their research team, who made it by the method announced in a preliminary communication (McCombie and Saunders 1946).

The action and toxicity of fluorophosphonates were studied in man by inhalation and in animals by inhalation as well as by intravenous or subcutaneous injection.

Inhalation in man

An approximately cubical steel-framed glass chamber of 10 cu.m capacity was used. The desired concentration of ester vapour was obtained by dissolving the calculated

weight of the compound in about 20 cc of ether and spraying it into the chamber by means of an atomizer worked by compressed air, mixing being achieved by three electric fans. After about 30 sec., the subjects, one to four in number, who had been waiting in an air-lock, walked into the chamber and quickly shut the door behind them. At the end of the exposure, the subjects left through the air-lock, and the chamber was cleared by a large suction fan. In the calculation of the concentration (e.g. 1 in 100,000) it is assumed that the ester is completely volatilized and that the gram-molecular weight occupies a volume of 22.4 litres.

Inhalation in animals

Static method—A wood-framed glass chamber of 1.78 cu.m capacity was used. A batch of animals (e.g., 3 rabbits, 4 guinea-pigs, 6 rats, and 10 mice) was placed inside in wire cages, the chamber sealed and the calculated amount of fluorophosphonate dissolved in 20 cc of ether sprayed into it. The exposure was timed from when about half of the material had been dispersed, an operation which usually took less than a minute. Mixing was achieved by an electric fan in the chamber. At the end of the exposure, an exhausting fan was switched on, the chamber opened and the cages removed by two operators wearing service respirators. The animals were removed to another room and kept under observation. Post-mortem examinations were made on animals that died.

Constant-flow technique—An apparatus was constructed containing an all-glass exposure chamber of about a litre capacity fitted with ground glass joints, in which either one rat or four mice could be placed. A constant stream of air, at a known rate of flow, was passed through this chamber, either by using compressed air which was passed through a gas-meter in series with the chamber or, in other experiments, by suction, measured amounts of water being run out of a 20-litre aspirator. All or part of the air stream could be passed through a bubbler containing diisopropyl fluorophosphonate, which was weighed before and after the experiment to determine the amount of ester volatilized. The rate of volatilization was also controlled by varying the temperature of the water-bath in which the bubbler was immersed. An identical bubbler containing sulphuric acid was fitted in parallel, and in an exposure pure air was passed through the chamber until conditions were steady and the animal calm, and then by means of a three-way tap the air stream was caused to pass through the ester for the desired time. In this manner it was possible to make 1-min exposures, because the desired concentration was rapidly attained at the beginning of the exposure, and the ester quickly swept out at the end. When only a part of the total air stream was passed through the bubblers, flow-meters were included in the circuit so that the air streams could be adjusted to give approximately the desired concentration of ester. The two air streams were allowed to mix in a suitable compartment before entering the exposure chamber. The bubblers, mixers and exposure chamber were connected by ground glass joints. When necessary, the gases leaving the exposure chamber were passed through towers containing activated charcoal or through service respirator canisters in order to remove the ester vapours.

RESULTS

Inhalation in man

The inhalation of air containing low concentrations of dimethyl, diethyl, or diisopropyl fluorophosphonate leads to effects qualitatively similar for all three esters, but varying in intensity with the nature of the ester dispersed, its concentration, and the time of exposure. Within a minute or so a feeling of tightness of the throat is noticed and respiration becomes slightly more laboured.

A few minutes later the pupils constrict to pin-point size and remain so for a long period, this causes the subject to experience the sensation that the room has dimmed, as though the sky had suddenly become heavily overcast. After a few hours, reading becomes almost impossible unless the book is held only a few inches from the eyes, at a distance of about 18 in print appears blurred. This effect must be due to a strong constriction of the ciliary muscles. The diisopropyl ester has the most powerful action of the three compounds. When two subjects were exposed for 3 min to a nominal concentration of 1 in 100,000 (82 mg/cu m) of this material, a tightness of the throat and slight difficulty in inspiration occurred during the exposure, and about 10 min. later the pupils constricted to pin-point size and remained so for days. The miosis subsided after about a week in the younger subject (28 years) and after two or three days in the elder (60 years). After about 24 hours there was eye-ache and headache which persisted for a day or two.

Inhalation in animals

Animals were exposed for longer periods and to higher concentrations than those used with man, and severe symptoms were produced, death frequently resulting. The effects were approximately the same in all species examined and with each of the three compounds. There was excessive salivation, nasal discharge, lacrimation and frequently pupil constriction, respiratory distress accompanied by intense gasping movements, and, in severely affected animals, convulsions leading to death, which usually occurred within about half an hour of the beginning of the 10-min exposure, and sometimes actually during the exposure. The rapidity of action of these materials as lethal inhalants is noteworthy and the majority of animals that survived half an hour usually made a complete recovery, although a few deaths occurred up to 24 hours.

The mortalities produced in rabbits, guinea-pigs, rats, and mice by 10-min exposures to various nominal concentrations of the three compounds are shown in Table I. For each experiment 3 rabbits, 4 guinea-pigs, 4 or 6 rats, and 4 or 10 mice were exposed in a static chamber. Of the four animal species, it will be seen that rabbits are the least and mice the most susceptible.

TABLE I

DEATHS RESULTING FROM 10-MIN EXPOSURE OF SMALL ANIMALS TO VARIOUS CONCENTRATIONS OF FLUOROPHOSPHONIC ESTERS

Compound	Methyl ester			Ethyl ester			Isopropyl ester	
Concentration	1/5,000	1/10,000	1/20,000	1/5,000	1/10,000	1/20,000	1/10,000	1/20,000
Rabbits	0/3	0/3	0/3	0/3	0/3	0/3	2/3	0/3
Guinea-pigs	3/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4
Rats	4/4	4/6	1/4	4/4	0/6	1/4	6/6	4/6
Mice	4/4	10/10	4/4	4/4	10/10	0/4	10/10	3/10

A more detailed study was made of the toxicity of the diisopropyl ester for rats and mice, the concentration necessary to kill 50 per cent of the animals exposed (LC50) was determined for each of five constant exposure times (1, 2, 5, 10, and 30 min) by the constant-flow technique. A convenient measure of the toxicity of a lethal inhalant is obtained by the product of the LC50 and the time of exposure in minutes (t). This value is referred to in the present paper as the LC t 50. Ideally it should be a constant and independent of the exposure time, but if detoxification takes place during exposure LC t 50 should increase greatly during long exposure to a low concentration, this is observed, for instance, when hydrogen cyanide is used as a lethal inhalant. The small changes observed in the values of LC t 50 in our experiments with fluorophosphonates, however, were probably not statistically significant, but a minimum value of the LC t 50 at 5–10 min might be indicated. With the static method an increase in LC t 50 with longer exposures may indicate that the concentration of toxic agent is falling off, but this effect can be eliminated by the constant-flow technique, in which a constant concentration is maintained by replacement. The LC t 50 values for rats using 10- and 30-min exposures were determined by both static and constant-flow methods, and almost identical values were obtained, indicating that no appreciable decay of concentration occurred in the static method up to 30 min, so that the nominal concentrations employed in the static method used to obtain the data in Table I are probably fairly close to the true concentration.

Estimates of the LC50 and LC t 50 values for rats and mice are shown in Table II. These estimates were obtained, by graphical interpolation, from the results of experiments in which at least 4 animals were used to determine each point on the mortality-concentration curves, in all, 197 rats and 390 mice were used.

TABLE II

LC50 AND LC t 50 VALUES FOR RATS AND MICE EXPOSED TO DIISOPROPYL FLUOROPHOSPHONATE
CONSTANT-FLOW TECHNIQUE

Animal	Exposure time min	Deaths within 2 hr		Deaths within 48 hr	
		LC50 mg./cu m	LC t 50 mg./cu m./min	LC50 mg./cu m.	LC t 50 mg./cu m./min
Rats	1	4,200	4,200	4,200	4,200
	2	2,000	4,000	1,800	3,600
	5	700	3,500	570	2,850
	10	360	3,600	280	2,800
	30	180	5,400	150	4,500
Mice	1	5,000	5,000	4,000	4,000
	2	2,650	5,300	1,900	3,800
	5	750	3,750	540	2,700
	10	440	4,400	350	3,500
	30	185	5,550	150	4,500

A more detailed analysis of the survival period of these animals shows that 55 per cent of the rats and 53 per cent of the mice had died within the first 2 hours after exposure, during the next 48 hours a further 15 per cent of the rats and 22 per cent of the mice died, there were no further deaths among the rats and only 1 per cent among the mice, 30 per cent of the rats and 23 per cent of the mice surviving. The rapid lethal action of fluorophosphonates becomes even more evident if the deaths occurring during the first two hours are grouped in half-hour intervals, if for this calculation the deaths after 30-min exposure are omitted, it is found that the majority of animals died within the first half-hour after exposure. Of the 108 rats dying within the first two hours, 95 died within the first, 10 within the second, and 3 within the third half-hour period after the beginning of exposure, the corresponding figures for 207 mice dying during the first two hours were 183, 10, 9, and 8 mice respectively for the four half-hour intervals.

Injection into animals

After injection of a solution of the diisopropyl ester in normal saline into the ear vein of rabbits there was excessive salivation, muscular twitchings, and loss of muscular co-ordination, sometimes urination and defaecation and usually convulsions prior to death. The pulse-rate was slowed like the respiratory-rate, but respiration ceased before the heart stopped beating. There was constriction of the pupils, commencing 2-5 mm after injection. The LD50 determined in a small number of rabbits was 0.5-0.75 mg/kg.

The LD50 for mice for subcutaneous injection of the diisopropyl ester dissolved in normal saline was 4 mg/kg, determined by graphical interpolation of the results of injections into batches of mice over a suitable dose range.

Atropine

Since it had been found (see Adrian, Feldberg and Kilby, 1946, 1947) that fluorophosphonates were extremely strong inhibitors of cholinesterase, experiments were made to determine whether atropine would be an effective antidote.

In rabbits intravenous injections of atropine were made either before or immediately after intravenous injection of a lethal dose of diisopropyl fluorophosphonate. When atropine was injected after the ester it was incapable of saving life, but when it was injected before the ester it appeared to reduce the death rate. For instance, when atropine (in doses between 4 and 50 mg/kg) was injected immediately after a lethal dose of diisopropyl fluorophosphonate (1 mg./kg.) the sole effect was to alleviate the severity of the symptoms and postpone death for a short time. This effect of atropine occurred only if the atropine was given immediately after the fluorophosphonate; if given only a few minutes later atropine had no alleviating effects whatever. When, on the other hand atropine (10 mg per kg.) was given intravenously 10 min before a lethal intravenous dose of fluorophosphonate (1 mg./kg.) the appearance of symptoms was not only delayed and their severity reduced, but the lives of some of the animals were actually saved, for instance, out of five rabbits so treated, three survived and the other two died only after 1½ to 3½ days, whereas all five control rabbits, given the fluorophosphonate without atropine, died within 45 min.

DISCUSSION

The toxic effects of the fluorophosphonates resemble in many respects those of eserine and prostigmine and are probably due to the anti-cholinesterase activity which these esters have been shown to exhibit (Adrian, Feldberg, and Kilby, 1946, 1947). Parasympathomimetic effects are very pronounced: the eye effects, the excessive salivation and lacrimation and the slowing of the heart may easily be explained as being due to accumulation of acetylcholine released from the parasympathetic endings. It is thus not surprising that these effects are alleviated by atropine. In addition the fluorophosphonates appear to have a definite "nicotine-like" action on skeletal muscle and on the central nervous system. The excitatory effects of fluorophosphonates on these structures are less pronounced, and the paralysing effects predominate. The fluorophosphonates share this predominance of a paralysing action with other anti-cholinesterases, and the problem of why under certain conditions some inhibitors of cholinesterase are mainly excitatory and others mainly depressant has never been satisfactorily explained (For review see Feldberg, 1945).

Death from fluorophosphonate poisoning probably results from respiratory failure, partly owing to obstruction of bronchioles, but mainly to paralysis of the respiratory centre; in this way too, the fluorophosphonates resemble other anti-cholinesterases. However no detailed analysis has been made of the exact nature of the cause of death.

SUMMARY

1 Inhalation of fluorophosphonates in man and animals leads to respiratory distress, pupillary constriction and spasm of accommodation. In animals, inhalation in higher concentrations than those used in man causes in addition excessive salivation, lacrimation, convulsions and death, probably owing to respiratory failure. Similar effects are observed in animals on intravenous injection of fluorophosphonates.

2 The percentage of deaths among small animals after 10 min exposure to various concentrations of dimethyl, diethyl, and diisopropyl fluorophosphonates is recorded. The concentration of diisopropyl fluorophosphonate which will kill 50 per cent of rats or mice exposed (LC₅₀) was found for 1, 2, 5, 10, and 30 min exposures. The product of the LC₅₀ and the time of exposure in minutes (the LCt₅₀) was found to vary between 2,700 and 4,500 mg/cu m/min. The LCt₅₀ showed a minimum for 5 to 10 min exposures.

3 Death, if it occurred, usually took place within the first half-hour after exposure.

4 The LD₅₀ of diisopropyl fluorophosphonate is about 4 mg/kg for subcutaneous injection in mice and 0.5–0.75 mg/kg for intravenous injection in rabbits.

5 Atropine given before the fluorophosphonate alleviated the severity of symptoms and reduced the death rate, but had little effect if given afterwards

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THE MODE OF ACTION OF MYANESIN

BY

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Berger and Bradley (1946, 1947) have shown that α β -dihydroxy- γ -(2-methylphenoxy)-propane ("myanesin") produces muscular relaxation and paralysis in experimental animals. The effects of the drug are of particular interest because paralysing doses of myanesin do not cause arrest of respiration. In this respect myanesin differs from curare and similar muscle-relaxing agents, which do not produce paralysis without simultaneous respiratory depression or arrest. It was therefore of interest to examine in greater detail the mechanism by which the effects of myanesin are produced. This report describes certain aspects of the action of the drug on voluntary muscle, the myoneural junction, peripheral nerves, and the central nervous system.

Action on the isolated voluntary muscle

The action of myanesin was investigated on the isolated rectus abdominis of the frog (*R. temporaria*) during November and December. The muscle was suspended in oxygenated Ringer's solution and arranged for recording the contractions on a kymograph. Concentrations of drugs were expressed as final concentrations in contact with the muscle. Myanesin at 1 in 750 or higher dilutions did not cause any effects. Stronger solutions such as 1 in 500 produced a slow contracture, which began about 10 minutes after the addition of the drug and gradually increased until about 1 hour later the muscle had shortened by about one quarter of the maximum possible contraction. At this stage the muscle still responded to electrical stimulation.

The effect of myanesin on the contraction produced by acetylcholine was also investigated. Acetylcholine, 1 in 100,000 produced almost maximum contraction, after 2 similar contractions had been produced, myanesin or other drugs were added and allowed to remain in contact with the muscle for 10 minutes. The muscle was then washed and the response to acetylcholine retested at suitable intervals until contractions were obtained similar to those before application of the drug.

Table I gives the reduction of the contraction expressed as the percentage of the contraction before administration of the drug. Myanesin 1 in 1,000 reduced the response to acetylcholine to about one half and 1 in 750 to about one third. Stronger solutions of myanesin were not used because they affected the muscle itself. Tubocurarine chloride and procaine hydrochloride, when similarly examined, were found to exert a very much stronger effect than myanesin. The

TABLE I

THE ANTAGONISTIC EFFECT OF MYANESIN AND TUBOCURARINE ON CONTRACTIONS OF THE
FROG'S RECTUS ABDOMINIS

Contractions were produced by acetylcholine 1 in 100,000

Drug	Dilution	Reduction of contraction as per cent of original contraction
Myanesin	1 2,000	25
	1 1,000	53
	1 750	68
Tubocurarine chloride	1 1,000 000	36
	1 750,000	46
	1 500,000	60
	1 100,000	93

concentrations of tubocurarine chloride, procaine, and myanesin causing approximately 50 per cent reduction in the response to acetylcholine were 1 in 700 000, 1 in 6,000, and 1 in 1 000 respectively

The experiments show that myanesin in high dilution does not antagonize the action of acetylcholine on voluntary muscle. It is therefore likely that myanesin produces its effects by a mechanism different from that of curare. Although myanesin and procaine are local anaesthetics of almost equal potency, procaine antagonizes the effects of acetylcholine more strongly than myanesin. This observation suggests that the curare-like action of local anaesthetics is an independent property of such drugs and may have little relation to their paralysing effects on nerve endings or nerve trunks.

Effect on peripheral nerves

The action of myanesin on the motor nerve was examined on a simple muscle nerve preparation of the frog. The sciatic nerve was immersed in a solution of the drug. Excitability was tested at the cut end of the nerve at 1 minute intervals with galvanic current from an induction coil. The time after which contraction of the muscle was abolished was noted. The action on sensory nerves was examined by the plexus anaesthesia method in frogs and the intracutaneous weal method in guinea-pigs as described by Bülbring and Wajda (1945). Cocaine or procaine were used as standards of comparison. All drugs were used at three dose levels.

TABLE II

LOCAL ANAESTHETIC POTENCY OF MYANESIN EXPRESSED IN TERMS OF PROCAINE AND COCAINE

Method	Number of animals used with myanesin	Number of animals used with procaine	Potency as percentage of that of procaine	Number of animals used with cocaine	Potency as percentage of that of cocaine
Motor nerve (frog)	12	12	98	12	35
Sensory nerve (frog)	12	12	96	12	39
Intracutaneous weal (guinea-pig)	18	18	69	—	—

The results are summarized in Table II. Myanesin had about the same potency as procaine and one third that of cocaine when tested on the motor and sensory nerve of the frog. When examined by the intracutaneous weal method in guinea-pigs, myanesin had about two thirds of the activity of procaine. The relatively weak local anaesthetic action of myanesin suggests that the paralysing effect of the drug is not due to a direct action on peripheral nerves.

The curare-like action

It has been shown previously that myanesin in large and nearly lethal doses can produce paralysis of the muscle to indirect but not to direct stimulation. This effect may be due either to a curare-like action at the myoneural junction or to a more central paralysis of the nerve-endings (Berger and Bradley, 1946). Further experiments were carried out to ascertain the importance of this effect in myanesin paralysis.

(a) *Experiments on cats*—Chloralosed or decerebrated cats were arranged for registration of contractions of the gastrocnemius muscle. The muscle was stimulated alternately directly and indirectly at 10 seconds intervals by single induction shocks. Intravenous injection of myanesin in doses up to 150 mg. did not effect the response to direct or indirect stimulation in any way. Tubocurarine chloride (0.5 mg.) abolished the response of the muscle to indirect stimulation but hardly influenced the response to direct stimulation. The experiments show that myanesin injected into cats in doses of 150 mg. does not possess curare-like action.

(b) *Experiments on mice*—White mice weighing 18 to 22 grams were injected intraperitoneally with myanesin, cocaine, or procaine. After 10 minutes they were anaesthetized with ether and decerebrated. The sciatic nerve was then exposed in the gluteal region and cut. Fifteen and thirty minutes after injection of the drug, the peripheral end of the nerve was stimulated with faradic current of increasing voltage and the presence or absence of contraction of the muscles of the leg was noted.

Myanesin in doses of 300 mg per kg (i.e., about 50 per cent of the LD₅₀) did not cause paralysis of the muscle to indirect stimulation. Larger doses such as 500 mg per kg (80 per cent of the LD₅₀) made even the strongest stimulation of the nerve ineffective after 30 min., but did not significantly alter the threshold to direct stimulation. The disappearance of the response to indirect stimulation could be caused by a depression of neuromuscular transmission or by paralysis of the nerve. It was of interest to ascertain whether other local anaesthetics such as cocaine or procaine, could paralyse motor nerves when administered in very large doses. Cocaine in doses as large as 100 mg per kg (LD₇₅) did not abolish or impair the response to indirect stimulation. Similar results were obtained with procaine. It does not appear possible to produce paralysis of peripheral nerves by the systemic administration of local anaesthetics such as cocaine or procaine. It is therefore unlikely that paralysis to indirect stimulation produced by large doses of myanesin could be due to a paralysing action on the nerve, particularly as myanesin is a less potent local anaesthetic than cocaine. Myanesin may have an effect on the nerve because of a selective affinity of the

nerve tissue for the drug, but no evidence in favour of this assumption is available. It appears more likely that myanesin can block neuromuscular transmission when administered in very large doses. This curare-like effect does not play any part in the production of reversible muscular paralysis with smaller doses because under such conditions paralysis to indirect stimulation was never observed.

The myanesin-strychnine antagonism

It has been shown previously that myanesin can antagonize the actions of strychnine (Berger and Bradley, 1946). In those experiments the drugs were administered subcutaneously in 2.5 per cent gum acacia solution. To measure the antagonistic effect more accurately a series of experiments was carried out in which the drugs were administered intravenously. With this mode of administration, variations due to differences in the speed of absorption were eliminated.

White mice weighing 18 to 22 grams were used. Injections were made into the tail vein at a rate of 0.3 c.c. per minute. The convulsant and myanesin were injected together in a volume of 0.4 c.c. per 20 grams body weight.

The minimal lethal doses (MLD), approximately equal to the LD₈₀, of strychnine sulphate and leptazol were 0.43 and 100 mg per kg respectively. The median lethal dose (LD₅₀) of myanesin was 320 mg per kg.

Mice were injected with single minimal lethal doses of strychnine or leptazol, or multiples thereof. Myanesin was administered simultaneously and, for each dose of the convulsant, doses of myanesin were found which protected some of the animals from death. From these values the dose of myanesin protecting 50 per cent of animals was found graphically by plotting the probits of the percentage mortality against the log doses.

TABLE III

STRYCHNINE-MYANESIN ANTAGONISM AFTER SIMULTANEOUS INTRAVENOUS ADMINISTRATION TO MICE

Strychnine dose as multiple of MLD	Myanesin		Ratio to number injected of number		Dose of myanesin protecting 50% of mice mg/kg
	mg/kg	fraction of LD ₅₀	convulsed	died	
1	—	—	35/35	30/35	12
1	40	1/8	0/10	0/10	
1	20	1/16	2/10	2/10	
1	10	1/32	9/10	6/10	
2	80	1/4	0/20	0/20	34
2	40	1/8	17/20	7/20	
2	20	1/16	10/10	9/10	
3	320	1	0/10	0/10	80
3	160	1/2	6/10	2/10	
3	80	1/4	10/10	5/10	
4	320	1	7/20	12/20	160
4	160	1/2	20/20	10/20	
4	80	1/4	10/10	10/10	

The antagonistic action of myanesin against leptazol was relatively weak. About one quarter of the LD50 of myanesin protected 50 per cent of the animals against 1 MLD of leptazol. The effect of myanesin in maintaining life was greater than its power to prevent the occurrence of convulsions. Protection against 2 MLD of leptazol could not be obtained even when administered together with one LD50 dose of myanesin.

The antagonistic action of myanesin to the effects of strychnine was well marked. Animals could be protected from the effects of one MLD dose of strychnine by as little as one thirtieth of the LD50 of myanesin, and proportionally larger doses were able to antagonize larger doses of strychnine (Table III).

When the median protective dose of myanesin was plotted against the dose of strychnine, expressed in terms of MLD, both values being plotted on a logarithmic scale, an approximately straight line was obtained (Fig 1).

In suitable dosage myanesin antagonized all the effects of strychnine, and animals to which balanced mixtures of the two drugs had been administered appeared quite normal. The myanesin-strychnine antagonism was more complete than the hexobarbitone-strychnine antagonism, in the latter it was not possible to find a balanced mixture, because doses of hexobarbitone which protected mice from convulsions and death caused a depression of greater duration and intensity in the presence of strychnine than in its absence.

Strychnine causes an increase of the reflex irritability of the spinal cord which may result in simultaneous contraction of all skeletal muscles if sufficiently large doses are given. Myanesin selectively antagonized the effects of strychnine in doses which by themselves did not produce any effects. It may therefore be inferred that myanesin decreased reflex hyper-

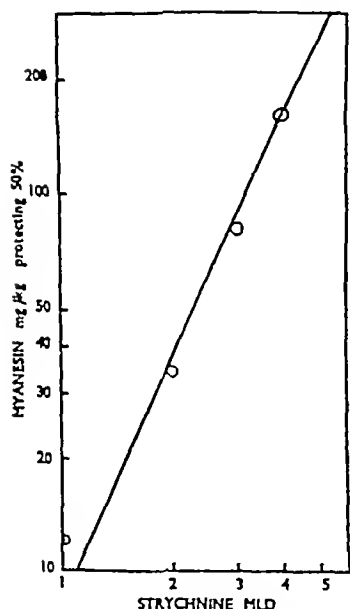


FIG 1—Antagonism of myanesin and strychnine. Abscissae: Dose of strychnine as multiple of MLD. Ordinates: Dose of myanesin protecting 50 per cent of animals.

excitability and prevented the passage of abnormal excitatory impulses through the reflex arcs.

Effect on tetanus

The effect of myanesin on experimental tetanus was examined in white mice. Ten micrograms of crude tetanus toxin were injected into the neighbourhood of the sciatic nerve high in the thigh. Local tetanus was apparent about 12 hours later and all animals died of generalized tetanus in about 3 days. Myanesin was administered intraperitoneally or subcutaneously at various times after tetanic spasms became apparent. In doses of

150 to 200 mg per kg. it completely abolished the spasm in all stages of the disease. The board-like rigidity of the extremities gave place to complete flaccidity a few minutes after injection of the drug. Myanesin in these doses did not cause depression of respiration or any other untoward effects and poisoned animals under the influence of the drug were indistinguishable from control animals to which myanesin only had been given. The effect of the drug lasted for about 30 min and was followed by a gradual reappearance of tetanus. Repeated doses of myanesin caused similar effects and regularly released the spasm. The impression was gained that the life of the animal could have been saved by continuous or frequently repeated administration of the drug.

The action of tetanus toxin on the central nervous system is similar to that of strychnine. The fact that myanesin can re-establish reciprocal innervation in conditions produced by these two different agents gives rise to the hope that it may also influence pathological excitatory innervation caused by degenerative processes of the central nervous system.

Effect on knee jerk

The effect of myanesin on the knee jerk was studied in cats. The animals were anaesthetized with chloralose intravenously and arranged for recording of the knee jerk as described by Schweitzer and Wright (1937). The limb was allowed to hang freely in order to facilitate observation of alteration in muscle tone. The jerk as a rule was elicited every 10 seconds.

Myanesin did not abolish the knee jerk of healthy cats. Intravenous doses ranging from 20 to 150 mg per cat either did not cause any alteration or somewhat reduced the height of the myographic record (Fig 2); the flexion at the knee joint was usually increased,

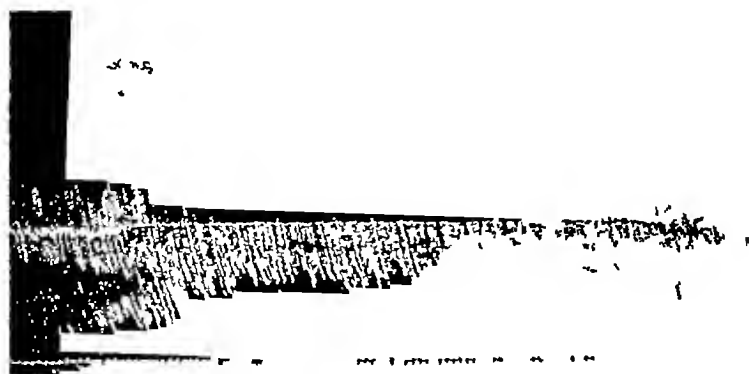


FIG 2—Cat 2.8 kg Chloralose (0.08 g per kg.) Record of knee jerk Time intervals 30 seconds At arrow 30 mg myanesin slowly injected intravenously

signifying a decrease in the tonus in the muscles, but complete inhibition of the jerk was not observed.

Some of the cats used in the experiments showed a very lively reflex followed by clonus. Exaggerated reflex excitability and tremors were also observed in certain animals under light chloralose anaesthesia (0.05 g chloralose per kg). Myanesin had a definite

effect on the knee jerk of these animals (Fig 3A and B and Fig 4). It inhibited clonus and tremors, abolished the irregular responses to patellar stimulation and reduced the knee jerk to its usual size. These effects could be produced with small doses of myanesin (20 to 30 mg. per 3 kg. cat). The effect set in almost immediately and lasted for about 20 minutes and sometimes longer.

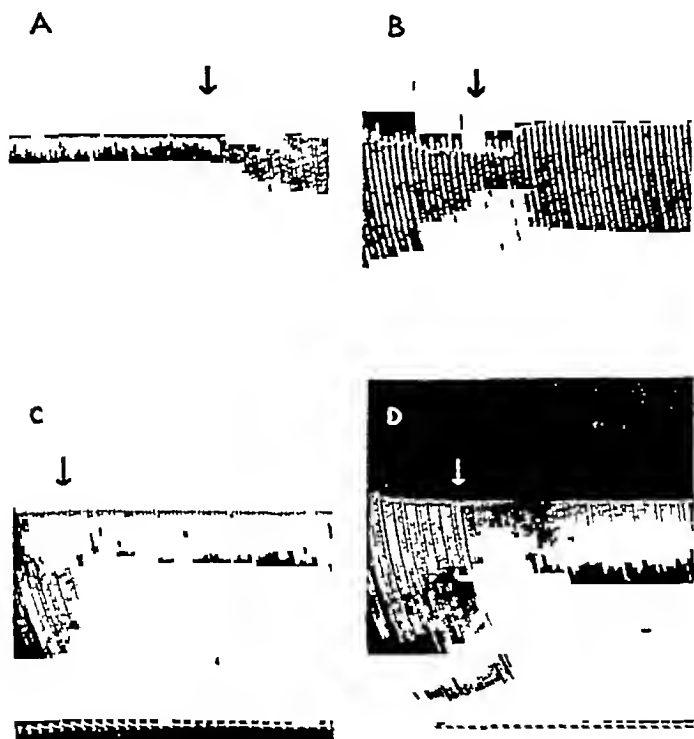


FIG 3—Records of knee jerk in cats weighing 2.8–3.1 kg. Jerk elicited every 10 seconds. Drugs injected intravenously: A Chloralose 0.05 g. per kg., myanesin 50 mg. B Chloralose 0.06 g. per kg., myanesin 150 mg. C Chloralose 0.06 g. per kg., strychnine 0.2 mg., myanesin 50 mg. D Chloralose 0.05 g. per kg., strychnine 0.4 mg., myanesin 100 mg.

The effect of myanesin on the experimentally increased knee jerk was also studied. Cats were injected with doses of strychnine insufficient to cause convulsions but producing exaggerated reflex activity. Myanesin in small doses promptly counteracted this increased reflex excitability and caused an immediate reduction of the reflex to the level present before administration of strychnine (Fig. 3C and D).

The effects of myanesin on the knee jerk of rabbits anaesthetized with urethane were also examined, they were similar to those observed in cats. Myanesin, 100 mg. per rabbit weighing about 3 kg., caused a decrease in the size of a few jerks immediately following the injection. Smaller doses did not influence the size of the jerk but abolished tremors, clonus and spontaneous movements of the leg.

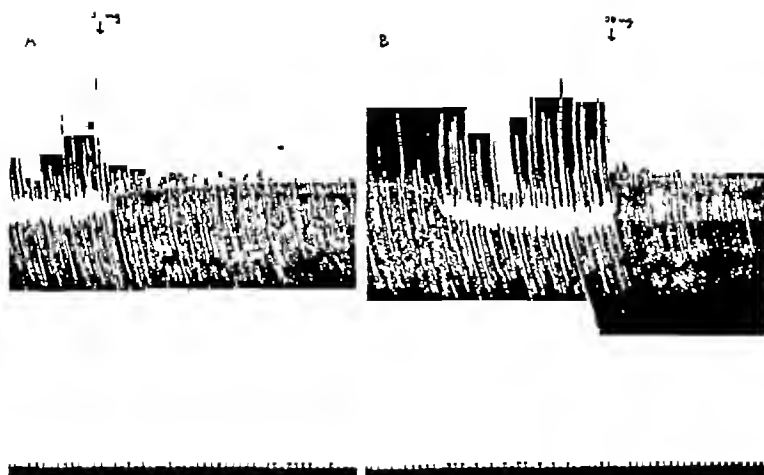


FIG 4—Record of knee jerk Cat 3.4 kg Chloralose 0.08 g per kg Time interval 30 seconds
At first arrow 30 mg myanesin, at second arrow 100 mg myanesin i.v. Between A and B a piece of the record occupying 40 minutes was cut out

The experiments show that although myanesin has little effect on the normal knee jerk of the cat, it is very effective in reducing an exaggerated reflex to its normal size. Myanesin in suitable doses may therefore exert an inhibiting action on pathologically exaggerated functions of the central nervous system without influencing normal reflexes.

DISCUSSION

The experiments reported in this paper show that the effects produced by myanesin are due to its peculiar action on the spinal cord. Myanesin selectively depresses hyperexcitability of spinal reflexes, but hardly influences the normal reflex actions mediated through the cord. Symptoms of hyperactivity, whether due to the action of poisons or to light anaesthesia, can be inhibited with smaller doses of myanesin than those required for the depression of normal physiological functions. Strychnine convulsions in mice can be counteracted with as little as 20 mg per kg intravenously, but 150 mg per kg or more, administered by the same route, are required to paralyse the animal. In cats 20 mg abolished tremors and clonus, but 150 mg did not abolish the knee jerk. The relation between the dose required to bring hyperfunction back to normal and that causing depression of normal function was approximately the same with mice, rabbits, and cats.

The depressant action of myanesin on the peripheral nerves (the local anaesthetic action) is too weak to play any part in the effects produced by systemic administration of myanesin. In concentrations which can be achieved after systemic administration, myanesin does not exert any direct relaxing action on

muscles and does not block the action of acetylcholine on them. The curare-like action obtained with large doses is of toxicological interest only and never becomes apparent with doses from which animals recover. It is incorrect to call myanesin a curarizing agent, because doses causing relaxation during anaesthesia do not influence neuromuscular transmission, but produce relaxation by a depressant action in the spinal cord.

Muscular relaxation produced by myanesin differs from that produced by curare not only in that the drugs act on different structures, but also in the order and degree in which various groups of muscles are affected. With curare, muscles with cranial innervation are affected first, the peripheral and intercostal muscles are paralysed next, and with complete curarization the diaphragm is affected to almost the same extent as other muscles. With myanesin the muscles of the posterior half of the body are affected first, next the peripheral and intercostal muscles, then the cranial muscles, the diaphragm is affected last and there is a distinct margin between doses causing muscular paralysis and those causing arrest of respiration.

Both curare and myanesin have been used for the production of muscular relaxation during anaesthesia (Mallinson, 1947). Curare is the agent of choice for complete suppression of respiration, such as is required during certain operations on the lungs. Myanesin, on the other hand, appears to be more useful for the production of muscular relaxation when suppression of respiration is not desired. Myanesin should never be used for the production of respiratory arrest, because the doses required for this purpose are large and affect the heart and blood pressure.

Occasionally when an attempt was made to produce respiratory arrest in an already paralysed rabbit by rapid intravenous injection of myanesin, a general rigidity without arrest of respiration developed. The rigidity, which in appearance was similar to decerebrate rigidity, lasted for about 1 min. and was followed by profound muscular relaxation from which the animals recovered. The cause of this symptom is not understood. It may be due to a direct action on the muscle. It was observed only in certain rabbits after rapid intravenous injection of concentrated solutions. It was not seen in anaesthetized animals. Other species of animals, as well as most rabbits, showed respiratory arrest if administration was continued after complete paralysis was obtained.

The rigidity observed after rapid intravenous injection to certain non-anaesthetized rabbits is the only sign of stimulation which has been observed with myanesin. With this exception myanesin caused pure depression in all species which were examined and at all dose levels. Crystalline *d*-tubocurarine chloride, on the other hand, may cause conspicuous signs of central stimulation such as trembling, hyperexcitability to stimuli, muscle twitching, and convulsions. These symptoms are particularly well marked in mice and rats, but can also be observed in other species (Cohnberg, 1946).

West (1935) observed that certain samples of curare removed the violent spasm of parathyroid tetany in dogs without paralysing the animal. This selective removal of pathological rigidities without apparent diminution of voluntary power was termed lissive action by West. Pure crystalline tubocurarine chloride possessed no trace of lissive power in dogs.

Experiments reported in this paper indicate that myanesin possesses a lissive action. It should, therefore, prove useful in the treatment of spastic paralysis, hypertonic states, and tremors. It may be expected that myanesin will be effective in these conditions in doses which do not affect consciousness, do not diminish muscular power, and do not cause side effects. Similar results may also be expected after oral administration because myanesin is well absorbed from the intestinal tract.

SUMMARY

1 Myanesin in high dilution had no direct action on the rectus abdominis of the frog and did not block the action of acetylcholine on this muscle.

2 The local action of myanesin on peripheral nerves was similar to that of procaine.

3 In doses causing reversible paralysis, myanesin did not possess a curare-like action, but toxic doses had a blocking effect on the myoneural junction.

4 Myanesin had but little effect on the normal knee jerk. An exaggerated knee jerk due to light anaesthesia, strychnine, or unknown causes was promptly reduced to the usual size.

5 Myanesin in small doses antagonized all the effects of strychnine and relieved tetanic spasm.

6 Myanesin had a selective depressant action on the spinal cord. In doses which had little effect on voluntary power it restored deranged reciprocal innervation to normal and counteracted symptoms caused by a release from inhibitions as observed during light anaesthesia. The powerful effect of myanesin on tremors, increased reflex excitability, and similar symptoms suggests that it may be useful in the treatment of spastic and hypertonic conditions.

I wish to express my thanks to the Directors of The British Drug Houses, Ltd., for their interest in the work and their permission to publish the results. My thanks are also due to Mr R. A. Hall and to Misses B. J. O'Brien, L. F. Carrick, D. M. Culver, D. M. Gurney, B. Hall, E. U. Haywards and M. E. L. Tattersall for technical assistance.

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THE USE OF DRUG ANTAGONISTS FOR THE IDENTIFICATION AND CLASSIFICATION OF DRUGS

BY

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A sensitive biological method for the identification of drugs has been described by Chang and Gaddum (1933). It consists in estimating the activity of an unknown substance in terms of a standard, quantitatively, in several different pharmacological tests. As a rule the ratio of activity between standard and unknown in different tests is only constant if the two samples are chemically identical, otherwise the ratio of activity in different tests varies. By this method even closely related substances may be differentiated.

Another biological method for the identification and differentiation of drugs consists in testing quantitatively their responses towards antagonists. It has been shown in a previous communication (Schild, 1947) that the effects of histamine and acetylcholine may be thus distinguished, since many antagonists although unspecific at high concentrations will differentiate between these drugs at lower concentrations. The present investigation is concerned with the question whether drugs which are more closely related than histamine and acetylcholine may also be differentiated in this way, or whether antagonists, even though they may be used in an exact and quantitative manner, would fail to discriminate between closely related substances.

If indeed some drugs, although distinguishable chemically or by their relative activity in different pharmacological tests, could not be distinguished by their reactions to antagonists, they might conveniently be grouped into a common pharmacological class. It will be shown that drugs can in fact be found which are related in this way, and that if a certain simple scheme of drug action be accepted a rational classification of active drugs based on their responses to drug antagonists may be attempted.

METHODS

The experiments were done on the isolated ileum of the guinea-pig, employing the apparatus for assaying antagonists on isolated tissues which has previously been described (Schild, 1947).

Plan of experiments—The object of these experiments was to find out whether drugs could be differentiated by their responses to antagonists. They were accordingly designed to test whether the effects of two given stimulant drugs would be reduced by some antagonist to the same extent or not.

The assays are performed in two stages. In a preliminary experiment doses of the two active drugs producing approximately equal submaximal effects are determined as well as a concentration of the antagonistic drug sufficient to reduce these effects without completely abolishing them. In a final experiment these drugs are administered in the presence and in the absence of the antagonist in a planned sequence, which must be statistically unbiased in order to allow a statistical analysis of the results of each experiment to be made.

A typical experiment is shown in Fig. 1. The order of addition of drugs to the bath is as follows. The doses are administered in sets of four, each set consisting of a random sequence of one dose of each of the active drugs alone and a further dose of each of the active drugs in the presence of the antagonistic drug (after a preliminary period of contact between tissue and antagonist of 2 min.) After each administration of the antagonist several doses of the active drug alone are interjected in order to let the tissue recover, if possible, from the preceding depression.

Persistent depression produced by antagonists is the most serious difficulty in planning an unbiased sequence of doses suitable for statistical analysis. The depressant effect is partly overcome by interposing doses of the active drug alone. More fundamentally, the arrangement of the experiment itself is such that each of the active drugs has an equal chance of being preceded by a depressant injection, and it is thus unbiased with regard to the main point at issue. It is merely a matter of convenience how many 'recovery' periods of active drug alone are interposed and it may in some cases be more advantageous to have a fixed small number of such periods rather than to wait until the response has reached a steady state.

A complete experiment consists of several sets (randomized groups) of four doses. The final effect is assessed by averaging the effects obtained in the individual sets. If the two drugs are depressed to the same extent by the antagonist, and \bar{y}_A and \bar{y}_B are the mean effects produced by drugs A and B, and \bar{y}_{AZ} and \bar{y}_{BZ} the mean effects produced by these drugs in the presence of the antagonist Z.

$$\begin{aligned} \bar{y}_A - \bar{y}_{AZ} &= \bar{y}_B - \bar{y}_{BZ} \\ \text{or} \quad \bar{y}_A + \bar{y}_{BZ} &= \bar{y}_B + \bar{y}_{AZ} \end{aligned}$$

The latter equation is tested statistically by an analysis of variance carried out as previously described (Schild, 1942). In practice there is usually some deviation from theoretical equality, but unless this is statistically significant it may be assumed that both drugs have been depressed to the same extent.

USE OF A DRUG ANTAGONIST FOR THE IDENTIFICATION OF AN UNKNOWN SUBSTANCE

Curare causes the release of a histamine-like substance from the perfused gastrocnemius muscle of the dog (Alam *et al.*, 1939). In the present experiment a dog's hind limb was perfused with defibrinated blood by means of a Dale-Schuster pump, the blood being oxygenated through the animal's own lungs, a solution of 50 mg crude curare was injected into the artery perfusing the limb and the effluent was collected. The blood was extracted by Code's method and the extract assayed on the guinea-pig's ileum. The apparent concentration

error Two of the mean squares are smaller than the error mean square and thus statistically obviously not significant. One of these is the mean square which assesses the relative reduction of the effects of histamine and the extract by benadryl Since there is no signifi-

TABLE I

ANALYSIS OF VARIANCE OF BENADRYL DEPRESSION OF HISTAMINE AND HISTAMINE LIKE SUBSTANCE RELEASED BY CURARE

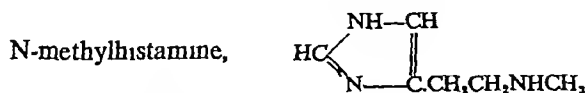
Source of variation	Sum of squares	Degrees of freedom	Mean square
Between successive "randomized groups"	4805	3	1602
Between histamine and extract	25	1	25
Between active drugs alone and active drugs + benadryl ('slope')	1764	1	1764
Between depression of histamine effect by benadryl and depression of extract effect by benadryl	6	1	6
Error	502	9	56
Total	7102	15	

cant difference the reduction of the two effects may be assumed to be equal and the main object of the experiment is achieved A further incidental result is a lack of statistically significant difference between the effects produced by histamine and the extract, indicating that the activity of the extract might reasonably be assumed to be 500 μ g per l. as originally assumed Two of the mean squares are much higher than the mean square for error and statistically significant One of these is the mean square for 'slope' indicating that the concentration of benadryl used produced a significant reduction of effect The other is the mean square between successive "randomized groups," showing that a real change in sensitivity, in this case a progressive deterioration, occurred in the course of the experiment.

Similar results were obtained in two further experiments

It was concluded that since benadryl does not discriminate between the two substances they are likely to be related or possibly identical

DRUGS WHICH ARE INDISTINGUISHABLE BY THEIR REACTION TO ANTAGONISTS



has been synthesized by Garforth and Pyman (1935) Its pharmacological actions were investigated by Vartiainen (1935) who found it to be twice as active as histamine on the guinea-pig's ileum and about half as active as histamine on the cat's blood pressure Since histamine and its N-methyl derivative are closely related, as well as clearly distinguishable by their relative activity in different pharmacological tests, they were selected as a representative pair to investigate the effect of antagonists on closely related substances

In the following experiments the effects of three different antagonists on the action of equiactive concentrations of histamine and N-methylhistamine were

studied. The N-methyl derivative was approximately 25 times as active as histamine in terms of molar concentrations. The antagonists used were antergan (N,N-dimethyl-N'-phenyl-N'-benzylethylene diamine hydrochloride, Halpern, 1942), pethidine, and atropine. The results are illustrated in Fig 2.

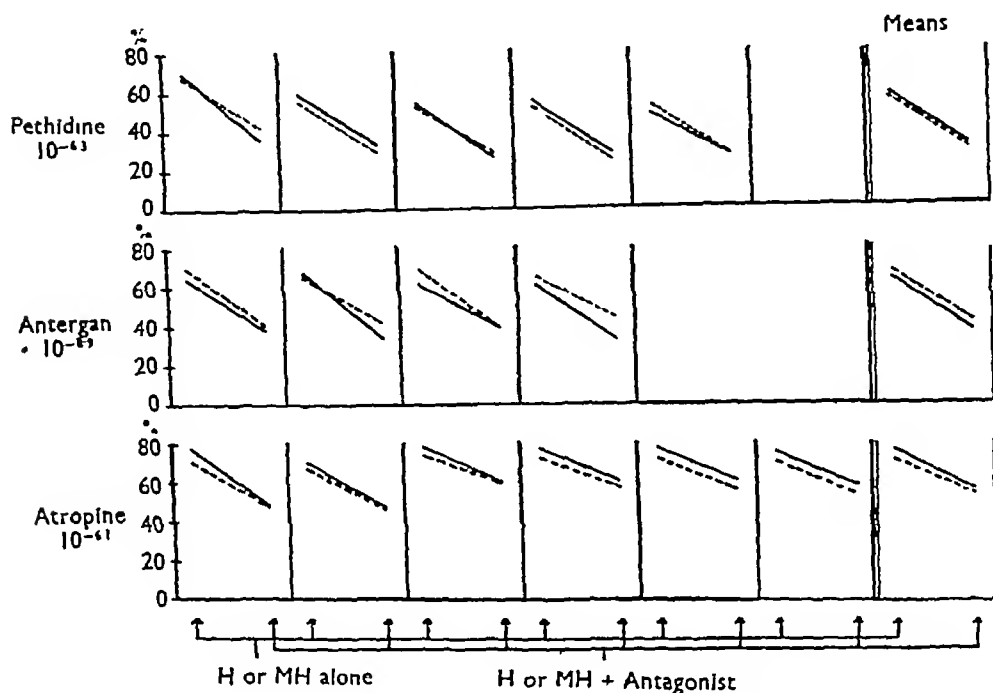


FIG 2.—Reduction of the effects of histamine (-----) and N-methylhistamine (——) by three different antagonists. The molar concentration of histamine used was approximately 2.5 times that of N-methylhistamine.

Points in the top left-hand corner of each square represent the effects of histamine or N-methylhistamine alone, the lower points the effect of these drugs in the presence of a constant concentration of antagonist. Successive squares in a horizontal row represent effects in successive "randomized groups" and the final square the arithmetical mean of effects, all obtained on the same strip of intestine.

The results were analysed statistically as in the previous example and it could be shown that a given concentration of each antagonist produced, within statistical limits, the same reduction of effect of the two stimulant drugs. It was concluded that none of the three antagonists is capable of discriminating between histamine and N-methylhistamine.

These experiments show that certain drugs react in an analogous manner towards antagonists of very different chemical and pharmacological nature. It is doubtful if any antagonist could be found to discriminate between histamine

and its N-methyl derivative. If the unknown substance discussed in the preceding section had in fact been N-methylhistamine it could not have been distinguished from histamine by means of these antagonists.

USE OF ANTAGONISTS FOR THE CLASSIFICATION OF DRUGS

It might reasonably be assumed that drugs which in a given pharmacological test could not be differentiated by their reaction towards antagonists, or, more precisely, drugs which in the presence of any effective antagonist showed equal reductions of equal effects, would be closely related in their pharmacological action on the particular test object used. Conversely, drugs which could be differentiated by their reactions towards most antagonists except the most unspecific ones might be said to be pharmacologically unrelated.

In thus using the effects of antagonistic drugs as a criterion for classifying active drugs, the following scheme of drug action might perhaps be visualized. It may be assumed that between the first impact of a drug on the tissue and its final effect, muscular contraction in this case, a series of successive processes occur with any of which antagonists can interfere. Two successive stages are indicated in Fig 3.

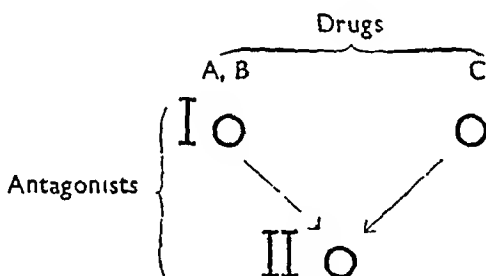


FIG 3—Site of action of antagonists. If A and B stand for histamine and N-methylhistamine and C for acetylcholine, it is possible that small concentrations of antagonists may act at one of the primary sites only, whilst larger concentrations may also antagonize a later common reaction.

If two drugs have different primary points of impact their reactions must sooner or later converge into a final common path. An antagonist may interfere with the action of a stimulant drug either before or after a common final path with some other drug is reached. Thus antagonist I reduces only the action of drugs A and B, but not that of C, whilst antagonist II reduces A, B and C. It may further be assumed at least as a first approximation, that an antagonist acting at a given site would reduce the effect of those drugs with whose pathways it interferes to the same extent. Thus antagonist I would depress A and B equally and antagonist II A and B and possibly C equally.

It follows that if two drugs such as A and B act by the same mechanism, their effects will be reduced to the same extent by antagonists, independently of whether the latter exert their action by competing for a primary site or by

interfering with some other reaction involved in the contractile process. Such drugs might well be classed together into a primary pharmacological class.

Antagonists may of course act at more than one site. A possible explanation for the dual action of drugs such as atropine and neo-atrergan (Schild, 1947), which antagonize both histamine and acetylcholine at relatively high concentrations, but only one of the two at low concentrations, would be that at low concentrations the antagonists act at the level of I only and at high concentrations at the level of II as well as of I. Another way of explaining the dual action would be to assume that at high concentrations neo-atrergan begins to affect the primary site for acetylcholine and atropine to affect the primary site for histamine. At any rate it is clear that at higher concentrations these antagonists must act on at least two sites.

The scheme is capable of expansion to include the action of further groups of drugs and their antagonists. A scheme of this kind cannot, however, account for special types of antagonism, such as a chemical combination between active drug and antagonist.

DISCUSSION

One of the earliest attempts at classifying drugs by their reaction to antagonists was the classification of substances contracting plain muscle into musculotropic and neurotropic according to their reaction to atropine. Various objections have been raised from time to time to this conception.

It was pointed out by Magnus (1905) that using the criterion of atropine to localize the site of action of drugs in the intestine implied firstly that atropine had only one site of action in the wall of the intestine and secondly that the effect of atropine could not be reversed by some other drug acting on the same site. Both these assumptions were unlikely to be true. It was indeed shown by Magnus himself that atropine had more than one site of action in the intestine and that it would antagonize at different concentrations drugs belonging to quite different groups such as pilocarpine and barium, and it had been shown by Langley and others that there existed a quantitative antagonism between atropine and pilocarpine. This line of criticism does not necessarily invalidate the use of drug antagonists for localizing the site of action of drugs, rather it points to the necessity of refining these methods by using drug antagonists in a more quantitative way. In practice the setting apart of a group of "muscarinic" drugs (Dale, 1914), exceptionally sensitive to atropine, has been extremely fruitful, and although the localization implied in the term "neurotropic" cannot be maintained any longer, these drugs must still be regarded as forming a group apart, likely to have a common mechanism of action.

From another point of view the old classification has been criticized by Winder *et al* (1946). These workers came to the conclusion that a subdivision into two groups was inadequate, since in addition to the acetylcholine group, a histamine and a barium group of plain muscle stimulants could be clearly dis-

tinguished by their differential reactions towards antagonists. There is, however, no special reason for confining plain muscle stimulant drugs to three types only and it would seem reasonable to look for further types to be differentiated by their reactions towards antagonists. This is precisely what the present classification proposes to do, since drugs which react quantitatively alike to antagonists are placed together, and drugs which can be differentiated by their quantitative response to antagonists are separated.

The first distinguishing feature of the proposed classification is that it relies upon a quantitative discrimination, the second that it relies upon the response to several antagonists rather than to a single one, only those drugs are assigned to a primary pharmacological class (in relation to a given tissue) which respond in a quantitatively identical manner to every effective antagonist. This method of classification has the merit of being sharply defined, but it may ultimately prove to have been too rigidly conceived. In a more general way, however, quantitative similarity in behaviour to antagonists is bound to denote some pharmacological relationship between drugs, and the recognition of these relationships may eventually lead to a better understanding of the mechanism of action of drugs.

SUMMARY

1 A method is described for evaluating statistically whether the effects of two drugs are reduced equally by antagonists.

2 If equal effects are produced on the guinea-pig's ileum by histamine and N-methylhistamine, they are antagonized to the same extent, quantitatively, by effective concentrations of three different antagonists. Similarly the effects of histamine and a histamine-like substance released from striated muscle by curare are equally depressed by an antagonistic drug. It is concluded that antagonists probably cannot be used to discriminate between closely related drugs.

3 A scheme of drug action is proposed which can serve as a basis for a classification of active drugs by means of drug antagonists.

I am indebted to Dr A. L. Morrison of the Roche Research Department for preparing N-N-dimethyl-N-phenyl-N'-benzylethylene-diamine, and to Dr Harold King for a supply of N-methylhistamine.

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THE INFLUENCE OF CALCIUM AND POTASSIUM IONS ON THE TOXICITY OF OUABAIN

BY

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In the mass of literature dealing with digitalis and Ca and K ions there are no quantitative results showing the influence of varying amounts of these ions on the action of a constant dose of glycoside, nor are there any laboratory observations on the effects of Ca or K on the symptom of digitalis vomiting. Accordingly the following studies have been made.

Anaesthetized rabbits have been used to determine (i) the lethal dose of ouabain infused intravenously at constant rate in physiological saline, (ii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of CaCl_2 , and (iii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of KCl.

Perfused rabbit hearts have been used to determine the effect of alterations in the amount of (a) CaCl_2 and (b) KCl in the Locke solution on the effect of a fixed concentration of ouabain.

Pigeons have been used to determine whether the injection of KCl modified the action of ouabain in causing vomiting.

EXPERIMENTAL RESULTS

(a) *Anaesthetized rabbits*

Method—Thirty-four animals were used. All were given urethane by ear vein till full surgical anaesthesia was reached, the dose necessary being remarkably constant at 1.55 g (6.2 ml of 25 per cent (w/v) in distilled water) per kg. of rabbit given over 10–15 min. Urethane was used in order to avoid ether, which increases the scatter of results of digitalis assay by the cat method (Burn, 1937), the quantity agrees well with the 1.8 g/kg. usually recommended for cats intramuscularly. Cannulae were inserted into the trachea, the left carotid artery for recording the blood pressure by mercury manometer, and the left femoral vein for the infusion of solutions. Artificial respiration was given by a pump as soon as an animal's respiration began to be shallow. Into all animals, except controls, ouabain (20 μg /ml) was infused in physiological saline at the constant rate of 0.15 ml/min/kg. rabbit. This rate of infusion is within the optimum range (Rapson and Underhill, 1935). Observations on the effects of Ca and K on the lethal dose of ouabain were made by adding varying amounts of CaCl_2 or KCl to the infusion and the end-point of an infusion was taken as the point at which blood pressure suddenly fell to zero without recovery. The animals were thus in the following groups

- (i) Receiving ouabain alone.
- (ii) Receiving ouabain + 1, 2 or 3 per cent (w/v) CaCl_2 .

(iii) Receiving ouabain + 0.5, 1 or 2 per cent (w/v) KCl

(iv) Controls receiving 3 per cent CaCl_2 alone

The control animals needed more than 0.5 g CaCl_2/kg to kill them, in agreement with Nahum and Hoff (1937) who gave 10 per cent CaCl_2 at 2 ml/min. In the present series none of the rabbits receiving ouabain and calcium received more than 0.09 g CaCl_2/kg . Since the effects of KCl on digitalis toxicity were protective, no controls with KCl alone were considered necessary.

Results—In order to compare the relative effects of CaCl_2 and KCl on the action of ouabain, concentrations were expressed as molarities, but percentage concentrations (w/v) were also recorded for convenience (Table I). The mean results are represented graphically in Fig. 1 in order to show the difference

TABLE I

RABBITS Femoral vein infusions of 20 μg ouabain/ml at 0.15 ml/kg/min

Fluid infused		Individual LD ouabain $\mu\text{g}/\text{kg}$ rabbit	Mean
Ouabain and CaCl_2 Amount of CaCl_2		30.5, 36.5, 35.7, 58.0, 58.0 60.0, 51.3, 73.5, 49.6, 74.7 75.5, 105, 93.3, 65.8, 56.0	43.7 61.8 79.1
Molarity $\times 10$	Per cent		
2.7	3		
1.8	2		
0.9	1		
Ouabain alone		114, 77.7, 81.0, 117, 88.0	95.5
Ouabain and KCl Amount of KCl		148, 105, 114, 123 121, 145, 132, 166, 141 171, 151, 156, 130	123 141 152
Molarity $\times 10$	Per cent		
0.67	0.5		
1.3	1		
2.7	2		

in slope of the curves relating mean LD ouabain (ordinates) to concentration of CaCl_2 or of KCl (abscissae) in the infusion. Clearly there is a qualitative difference in effect, increased CaCl_2 causing a linear decrease in LD ouabain, while increase of KCl causes an increase in LD ouabain, there also appears to be a quantitative difference, because the curve for KCl is the steeper. With the two lowest concentrations of potassium used, unit change of concentration had a greater effect on the LD of ouabain than had unit change in concentration of CaCl_2 , thus, 1, 2, and 3 per cent CaCl_2 decreased the LD ouabain by 17, 36, and 54 per cent respectively, whereas 0.5, 1, and 2 per cent KCl increased it by 29, 47, and 59 per cent. This suggests that the action of ouabain is influenced more by the absolute concentration of potassium in a perfusion fluid than by the potassium/calcium ratio, at least over a certain range. In order to

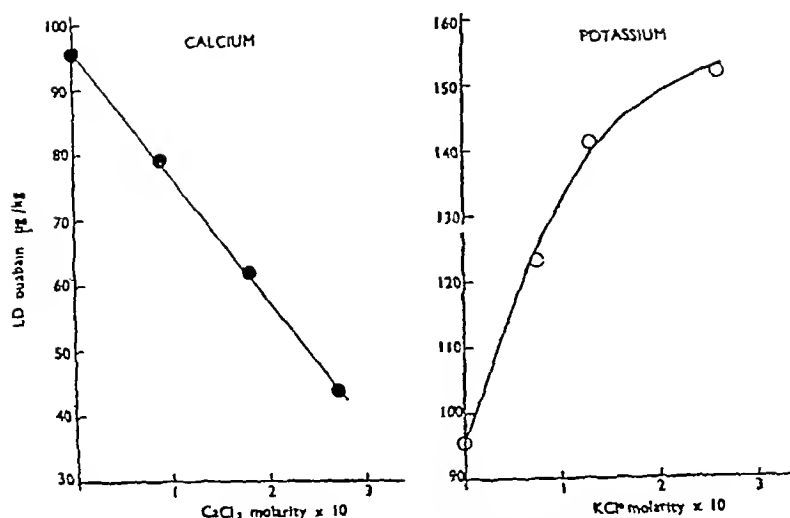


FIG 1—Rabbits Femoral vein infusion of ouabain (20 µg./ml) at a rate of 0.15 ml/kg/min. The relation between the LD ouabain and the Ca or K content of the infusion

test this point it was necessary to perform experiments in which potassium or calcium concentrations could be lowered as well as raised, and accordingly the isolated perfused rabbit heart was used

(b) Perfused rabbit hearts

Method—Thirty-six animals were used. Animals were killed by a blow on the head and bled out by cutting the throat. The heart was rapidly cut out, dissected clean and perfused with oxygenated Locke's solution (percentage composition (w/v) as follows: 0.9 NaCl, 0.042 KCl, 0.024 CaCl₂, 0.1 dextrose, 0.05 NaHCO₃) at 36° by the Martin-Langendorff method. A hook was passed through the apex of the ventricles and the amplitude of beat recorded on a smoked drum by a lever and writing point. Rate and amplitude of beat and coronary flow were allowed to become steady and from 30–45 min after setting up the preparation perfusion was commenced with ouabain (0.4 µg/ml) in the Locke's solution. The measure of toxicity of the ouabain was taken as the time it took to reduce the amplitude of beat to 50 per cent of its original value. This time is referred to as the survival time. Thus anything which increased the toxicity of the ouabain shortened the survival time, and anything which decreased the toxicity lengthened the survival time. All hearts except the controls received 0.4 µg ouabain/ml, and were divided into the following groups:

- (i) Receiving ouabain in normal Locke's solution
- (ii) Receiving ouabain in Locke with half or twice the normal CaCl₂
- (iii) Receiving ouabain in Locke with 0.5, 1.25, and 1.5 times the normal KCl
- (iv) Control hearts receiving no ouabain. With the highest and lowest concentrations of CaCl₂ or KCl used all hearts survived for long periods
- (v) Hearts in normal Locke containing 0.4 µg ouabain/ml, in which toxic effects were caused to disappear by changing to perfusion fluid containing excess KCl

Results—For the reasons already given, the concentrations of CaCl_2 and KCl were expressed in molarities. These results (Table II) are plotted graphically (Fig. 2) as mean survival times against molar concentrations of CaCl_2 or KCl , and, as for the anaesthetized rabbits, it will be seen that when potassium is increased above normal, unit change in concentration of potassium produces a bigger effect on survival time than unit change in calcium concentration when calcium is decreased. This is expressed more clearly (Fig. 3), when the same

TABLE II
RABBIT HEARTS perfused by Langendorff's method with $0.4 \mu\text{g}$ ouabain/ml.

Concentration in Locke CaCl ₂				Ratio	Individual survival times <i>in minutes</i>	Mean survival times <i>in</i> minutes
Molarity × 10 ³	mg per 100 c.c	Molarity × 10 ³	mg. per 100 c.c			
2.16	24	5.63	42	2.61	32, 39, 49, 50, 51, 34	38
4.32	48	„	„	1.30	23, 28, 14, 40, 13	24
1.08	12	„	„	5.22	60 39, 45, 28, 49	44
2.16	24	2.81	21	1.30	20, 13, 14, 16	16
„	„	7.03	52.5	3.26	92, 110, 45, 48, 66	72
„	„	8.44	63	3.91	87, 114, 195, 207, 254	171

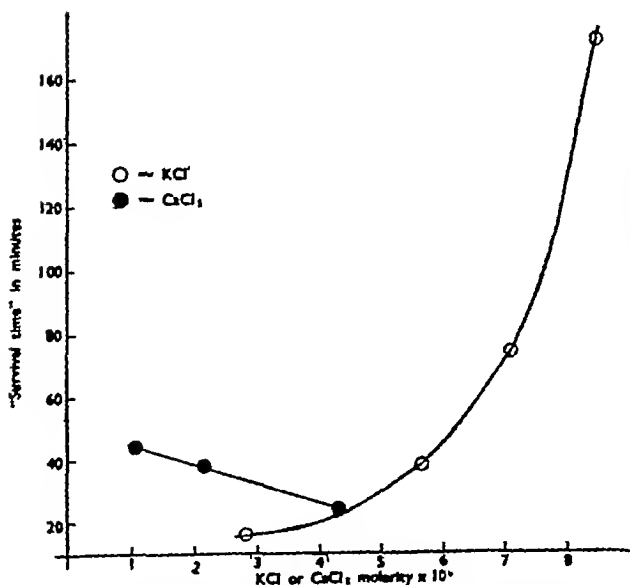


FIG. 2.—Langendorff rabbit hearts, $0.4 \mu\text{g}$ ouabain/ml Locke's solution at 36°C . The relation between the time required to reduce the amplitude of the beat to 50 per cent of its original value ("survival time") and the Ca or K content.

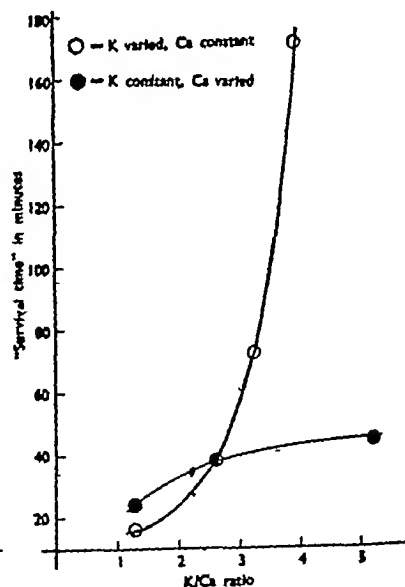


FIG. 3.—Same as Fig. 2. The relation between "survival time" and the K/Ca ratio.

survival times are plotted against the K/Ca ratio in the perfusion fluid. If the alteration in the ouabain effect had been solely a function of the K/Ca ratio, the same effect would have been obtained at a given K/Ca ratio whether it was attained by increasing K, or by decreasing Ca, but as Fig. 3 shows the absolute concentration of K had a relatively greater importance.

In addition to the quantitative results with potassium, three hearts were perfused with $0.4 \mu\text{g}$ ouabain/ml in normal Locke until gross irregularities of beat occurred, usually within 25 min., potassium was then added to the perfusion fluid to increase its KCl content by 50 per cent. This abolished the irregular rhythms caused systole to lessen, and prolonged the survival time, which would have been an hour at most, to several hours. A record of one of these hearts is shown in Fig. 4, and is in agreement with the results of Sampson *et al* (1943) on the human being.



FIG. 4—Langendorff rabbit heart. KCl abolition of irregular rhythm due to $0.4 \mu\text{g}$ ouabain/ml. Tracing reads from left to right. Time marker in minutes. Between (i) and (ii) ouabain added to the Locke to contain $0.4 \mu\text{g}$ /ml. Between (ii) and (iii) KCl concentration raised by 50 per cent, with abolition of irregular beats (iv) and (v).

(c) Pigeon emesis

Apparently the effect of K on the vomiting produced by digitalis bodies has never been investigated, except for the observation of Sampson *et al* (1943) that the nausea and vomiting of their patients who received an overdose of digitalis was not affected by potassium acetate administered orally. The pigeon-emesis method of digitalis assay introduced by Hanzlik and Schoemacher (1926), when modified as Burn (1930) suggested, affords a convenient method of testing this action of K against digitalis, if K were to lessen digitalis vomiting, this clearly would indicate antagonism of an extra-cardiac effect of digitalis, since digitalis vomiting has been shown to be independent of cardiac connections with the C.N.S. (Hanzlik and Wood, 1929, Haney and Lindgren, 1942).

Methods—Twenty pigeons were injected with $15 \mu\text{g}$ ouabain/300 g. pigeon into the wing vein on two separate occasions. The same pigeons were injected on two other occasions (interpolated between these two) with $15 \mu\text{g}$ ouabain and 6 mg. KCl per 300 g. pigeon. This amount of KCl was found to be about the maximum which pigeons would tolerate, for in a trial with KCl alone it killed one bird in a group of 20. The number vomiting is recorded in Table III. When the pigeons were injected with ouabain alone 22 out of 40 injections caused vomiting, with ouabain and KCl, 19 out of 40 injections caused vomiting. Clearly potassium did not protect pigeons against the vomiting induced by the ouabain.

TABLE III
PIGEON EMESIS RECORD

Pigeon number	Ouabain 15 μ g./300 g.		Ouabain 15 μ g. and KCl 6 mg /300 g	
	24 3 47	2 4 47	27 3 47	31.3 47
1	+	+	+	+
2	+	+	+	+
3	0	+	0	0
4	0	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	0	0	+
8	+	+	0	+
9	0	0	0	0
10	+	+	0	+
11	+	+	0	+
12	0	0	+	0
13	0	0	0	0
14	0	0	0	+
15	0	0	0	0
16	+	+	+	0
17	0	0	0	+
18	0	+	0	+
19	+	0	0	0
20	0	+	0	0
Total vomiting	10	12	7	12

DISCUSSION

Although there was reliable information concerning the effects of cardiac glycosides on frog hearts by the middle of the nineteenth century (Vulpian, 1855), it was not until after Ringer's proof of the importance of certain ions in the perfusion fluid (Ringer, 1883) that calcium and potassium became suspected of playing a part in the action of these glycosides. In frog hearts either Ca excess or K deficiency increased the action of digitalis, while Ca deficiency or K excess lessened its effect (Werschinn, 1910, Clark, 1912, Korschegg, 1913). From these results it was concluded that digitalis antagonized K just as Ca did. Some extreme views emerged, while Burrige (1915-16) postulated that the cardiac glycosides act by sensitizing the heart to calcium, Weizsacker (1917) drew a similar conclusion that digitalis improves the force of the heart only when there is a lack of calcium. Loewi (1918) reiterated Burrige's contention, and others wrote in support (Geiger and Jarisch, 1922, Grumach, Grünwald, Handovsky, Hoffmann, Schoen, 1923). Pietrkowski (1918), however, maintained that the effects of low Ca on the action of digitalis could be countered by increasing the sugar of the perfusion fluid, and concluded that digitalis has a direct action on the heart. All these observers were concerned with the systolic action of digitalis, and with the systolic effect of calcium, whereas it has been shown (Werschinn, 1910, Cushny, 1925) that small doses of digitalis result in

diastolic arrest Recently this observation has also been brought into line with the theory that digitalis sensitizes the heart to calcium (Blumenfeld and Loewi, 1945)

In mammals the occurrence of calcium and digitalis synergism has been well established both in animal experiments (Lieberman, 1932, Schunterman, 1935, Gold and Kwit, 1937, Gold and Edwards, 1927, Bower and Mengle, 1936, Golden and Brams, 1938), and in the human being, Edens and Huber (1916) finding that patients prone to digitalis pulse bigeminy have a high blood calcium, while Bower and Mengle (1936) record two cases of sudden death after intravenous calcium salts following digitalis

In contrast to the foregoing results are those of Fischer (1928) who found that digitalis sensitizes the heart to all stimuli, e.g., Ca, ethyl alcohol, or K, and of Camp (1939) who also found the heart after treatment with digitalis to be sensitized to K Nahum and Hoff (1937), however, and Smith, Winkler, and Hoff (1939) failed to obtain Ca and digitalis synergism Nyiri and DuBois (1930) disagree with the extreme view of Loewi, maintaining that digitalis can exert its full effect in the complete absence of calcium in the fluid perfusing a frog's heart, though they agree that excess of calcium enhances its action

A new line of evidence that K is involved in digitalis action arises first from the work of Calhoun and Harrison (1931) They showed that toxic doses of digitalis lower the level of cardiac K, the effect of therapeutic doses was doubtful Any theory based on these results which suggests that digitalis acts by lowering the K/Ca ratio appears to be untenable, since Calhoun and Harrison also found, in fatal human cases with cardiac failure, that the myocardium of the dilated chambers was low in K Confirmation of this effect of toxic doses has been obtained (Wood and Moe, 1938, Hagen, 1939, Wedd, 1939, Boyer and Poin-dexter, 1940) though these authors find the effect of therapeutic doses on K content is either negligible or else is to increase it K loss by digitalis action is also recorded from frog skeletal muscle (Cattell and Goodell, 1937), while further evidence of an effect of digitalis on K metabolism in general is given by Zwemer and Lowenstein (1940), who found that digitalis lowers the plasma K and prolongs life in adrenalectomized animals, thus calling attention to the chemical similarity between the digitalis bodies, especially digitoxigenin, and cortin Dorfman (1940), however, using adrenalectomized mice, was unable to demonstrate any cortin-like activity of strophanthin

Therapeutic advantage has been taken of the antagonism between potassium and digitalis (Sampson, Albertson and Kondo, 1943) in order to alleviate the cardiac effects of overdosage of digitalis by giving oral doses of potassium acetate No relief of nausea and vomiting was obtained but visual disturbances disappeared

The most recent publications concerning digitalis and heart biochemistry (Chen and Geiling, 1947) show that toxic doses of digitalis diminish cardiac

adenosine triphosphate (ATP), phosphocreatine and adenylic acid, while therapeutic doses have no such effect, and in decompensated hearts the re-synthesis of these three substances was hastened by digitalis administration (Weicker, 1935). These facts, together with the observation that the isolated perfused rabbit heart in systolic contracture from digitalis can be temporarily restored by ATP (Chen and Geiling, 1946), tempt speculation that the K/Ca effects of the cardiac glycosides may exert an influence on the intricacies of the higher energy-liberating phases of the chemical reactions concerned with muscle contraction.

The experiments now described give results which agree with those of the majority, increased calcium potentiates, and increased potassium antagonizes digitalis glycosides. The quantitative aspect takes the matter further, demonstrating the relative importance of the absolute concentration of potassium in the cardiac action of the glycosides, while the failure to protect pigeons against their emetic effect suggests that different biochemical mechanisms are concerned in the cardiac and emetic actions at least.

The ability of potassium salts not only to delay the toxic action of digitalis, but also to remove toxic effects when already developed is not generally known. Sampson and his colleagues (1943) gave 5-10 g potassium acetate by mouth as a 25 per cent solution to a series of 14 patients in whom digitalis had produced ectopic beats which were recorded by the electrocardiograph. Only one dose was given on any one day. The authors followed the rise in serum potassium and observed the disappearance of the ectopic beats. This occurred in every patient and outlasted the change in serum potassium. The observations described in this paper add support to these findings and suggest that they are due to the interplay of potassium and digitalis in the heart muscle itself.

SUMMARY

1 The LD of ouabain by intravenous infusion in physiological saline was determined on 34 rabbits under urethane.

2 The effect of CaCl_2 and of KCl on the LD of ouabain was observed by adding them to the perfusion fluid. 1, 2, and 3 per cent solutions of CaCl_2 decreased the LD of ouabain by 17, 36, and 54 per cent respectively, 0.5, 1, and 2 per cent solutions of KCl increased the LD by 29, 47, and 59 per cent respectively.

3 Survival times were observed of 36 Langendorff rabbit hearts perfused at 36°C with Locke's solution containing $0.4\text{ }\mu\text{g}$ ouabain/ml. The calcium or potassium concentration was varied in different experiments.

4 Increased calcium or decreased potassium shortened survival time, while decreased calcium or increased K lengthened it.

5 The effects of altering the potassium were greater than those of corresponding changes in calcium. Halving the CaCl_2 prolonged mean survival time by 6 min, increasing the KCl by 50 per cent prolonged it by 133 min.

6 In a group of 20 pigeons injected via the wing vein with ouabain (15 μ g /300 g pigeon) on two occasions, 22 out of 40 injections caused vomiting. On two other occasions with the same dose of ouabain plus KCl (6 mg /300 g pigeon), 19 out of 40 injections caused vomiting. The difference is not significant.

I wish to thank Prof Burn for the guidance and advice he has given throughout

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THE BLOCKING EFFECT OF BIS-TRIETHYL-AMMONIUM SALTS ON TRANSMISSION IN THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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The action of the tetra-ethylammonium ion on the circulatory system was described by Burn and Dale (1914) as a paralysing action on sympathetic ganglia resembling that of nicotine, the same type of action was observed in a series of triethylalkylammonium salts by Hunt (1925-6). The action of tetra-ethylammonium bromide on the circulatory system as well as on the sympathetic ganglion has recently been analysed by Acheson *et al* (1946). They concluded that the predominant effect of the tetra-ethylammonium ion was a block of transmission across autonomic ganglia and that this was sufficient to explain the vaso-depressor effect. Dr H R Ing suggested to us that it might be worth investigating the effect on ganglionic transmission of bis-triethylammonium salts of the general formula $[Et_3N(CH_2)_nNEt_3]X_2$, where X is the anion, such salts might be expected to have a blocking action on transmission in virtue of the triethylammonium groups and their potency might be expected to vary with the length of the polymethylene chain. Four bis-triethylammonium salts have been examined: ethylene bis-triethylammonium bromide (BTE2), prepared by Dr H R Ing, and the trimethylene-, pentamethylene-, and decamethylene-bis triethylammonium bromides, denoted by BTE3, BTE5, and BTE10 respectively, which were prepared by Mr R B Barlow.

METHOD

Cats were anaesthetized with pentobarbitone and the superior cervical ganglion was prepared by Kibjakow's method (1933) modified by Feldberg and Gaddum (1934). Warm oxygenated Locke's solution was perfused through a cannula in the carotid artery at a pressure of about 120 mm of mercury and the venous outflow from the ganglion was collected. The pre-ganglionic fibres to the superior cervical ganglion were stimulated maximally at a rate of 8 stimuli per second for a period of 15 sec. An interval of 3 min was allowed between each stimulation. The contraction of the nictitating membrane was

*Working with a grant from the Spanish Council of Scientific Research

recorded on a smoked drum by an isotonic lever. The activity of the four bis triethylammonium compounds was compared with that of tetra-ethylammonium bromide (TE). Each drug was given in 0.1–0.2 ml Locke's solution and was injected into the arterial cannula 1 min before the stimulation. A cumulative effect by the drug is very liable to occur when the perfusion rate becomes slow. It was therefore decided to test the drugs in one order and then in the reverse order.



FIG 1—Comparison of blocking effect of tetra-ethylammonium bromide (TE) and of two bis triethylammonium bromides (BTE2 and BTE3) on transmission in sympathetic ganglia. Perfused superior cervical ganglion of the cat. Record of the contractions of the nictitating membrane to maximal preganglionic stimulation. Rate of stimulation 8 per sec for 15 sec every minute. Figures above each contraction = outflow in drops per minute.

RESULTS

Fig 1 shows the effect of BTE2 and BTE3 in comparison with that of TE on the response to preganglionic stimulation in the same preparation. The figures above each contraction of the nictitating membrane represent the venous outflow in drops per minute.

It was found that the blocking effect of the five compounds examined was in the order

$$\text{BTE10} > \text{TE} > \text{BTE2} > \text{BTE5} > \text{BTE3}$$

$$(200) \quad (100) \quad (33) \quad (5) \quad (4)$$

The relative potency of these five compounds (according to their weight) has been determined on seven preparations, and, giving TE a value of 100, the mean

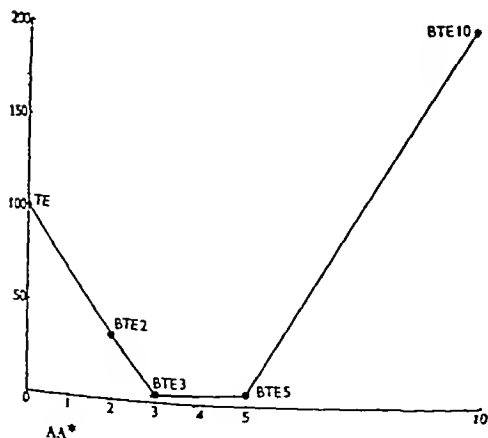


FIG 2—The relationship between the activity of tetra-ethylammonium bromide (TE), given a value of 100, and of bis triethylammonium bromides (BTE2, BTE3, BTE5, and BTE10) in the perfused sympathetic ganglion of the cat. Ordinate: percentage potency. Abscissae: Number of carbon atoms in the polymethylene chain of the molecule.

figures are expressed in brackets. No excitatory action on the ganglion was observed with TE, nor with the four bis-triethylammonium compounds.

Fig. 2 shows the relationship between the activity of these compounds and the number of carbon atoms in the polymethylene chain of the molecule.

DISCUSSION

In their two successive papers, Acheson *et al* (1946) concluded that the tetra-ethylammonium ion exerts a purely blocking effect on ganglionic transmission. Unlike tubocurarine, the tetra-ethylammonium ion on injection never causes a contraction of the nictitating membrane, nor does it increase the response to electrical stimulation, as intocostin was shown by Acheson *et al* to do. It has only a "nicotine-like paralyzing" action on the autonomic ganglion. The bis-triethylammonium compounds also showed no excitatory action. No experiments have been made in order to discover whether they have any muscarine actions. In general, the action of these compounds is very similar to that of tetra-ethylammonium salts. As shown in Fig. 2, lengthening of the carbon chain between the two onium ions leads to a decrease in activity until the three carbon chain compound (BTE3) is reached, with a further lengthening of the carbon chain there occurs an increase of the blocking effect, and the 10 carbon chain compound (BTE10) is twice as strong as TE.

SUMMARY

Four bis-triethylammonium compounds have been tested on the perfused superior cervical ganglion of the cat. They have no stimulating action, but they paralyze ganglionic transmission. There is a relationship between the relative activity of these compounds and the number of carbon atoms in the polymethylene chain of their molecules. The decamethylene-bis-triethylammonium bromide (BTE10) was found to be the most potent compound.

We wish to thank Prof. J. H. Burn and Dr. Edith Bülbürg for their guidance and encouragement in this work.

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THE PERSISTENCE IN THE BLOOD STREAM OF SOME ANALOGUES OF SULPHADIMETHOXYPYRIMIDINE

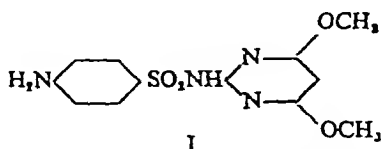
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An account has already been given of the properties of sulphadimethoxy-pyrimidine (I), which displays marked persistence in the blood stream after oral or parenteral administration (Gage *et al*, 1947)



The availability of some of its homologues (Rose and Tuey, 1946) prompted a comparative examination of their persistence, further compounds were specially prepared by Drs F L Rose, E H Hoggarth, and E H P Young. Twenty compounds in all were examined in mice for absorption and persistence. The majority were also tested as antibacterial agents, an account of their tuberculostatic activity *in vitro* and *in vivo* will be published separately (Hoggarth, Young, and Martin, 1948). No compound appeared likely to have marked therapeutic value. Three (III, VII, and XVI), as well as sulphadimethoxypyrimidine itself, had previously been examined by van Dyke *et al* (1945), their findings in general parallel those described here.

EXPERIMENTAL SECTION

The standard techniques used have been described in previous publications (Rose and Spinks, 1946, 1947, Gage *et al* 1947). Each compound was administered orally to a group of three mice as a 1 per cent (w/v) solution of the sodium salt, or as a 1 per cent (w/v) dispersion, in doses of 250 mg/kg. It was then estimated in pooled tail blood, at standard intervals after dosing, by the micro method of Rose and Bevan (1944). At least six groups of three mice were used for each compound (except XXI). No statistical comparison was attempted, because many compounds were so highly persistent that the maximum concentration was difficult to determine accurately in individual experiments. Values of maximum

concentration (Max), the time after dosing at which this was attained (t_{max}), and the persistence in the blood (expressed as the time (C7) taken for the concentration at 7 hours to fall to two thirds of that value), were read from the mean blood concentration time curves

TABLE
COMPARISON OF ANALOGUES OF SULPHADIMETHOXYPYRIMIDINE

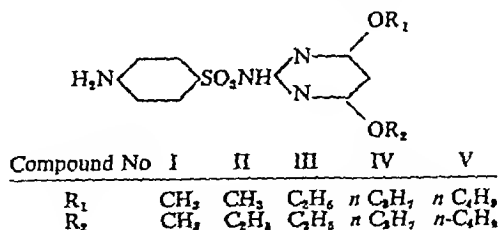
Group	Compound No	Max blood concentration		C7 (hours)	Number of mice
		mg /100 ml	Time (min)		
A	I	14.4	210	16.5	66
	II	13.8	100	15.3	18
	III	11.8	90	13.0	18
	IV	5.8	540 (?)	> 17.0	24
	V	2.2	180	7.8	21
B	VI	5.7	180	10.4	21
	VII	2.1	210	17.0	18
C	VIII	4.6	150	11.0	39
	IX	2.3	210	10.5	18
	X	5.7	270	9.0	30
D	XI	12.6	90	4.7	18
E	XII	24.7	90	7.7	30
	XIII	5.7	120	8.6	27
	XIV	5.9	40	6.0	18
F	XV	13.8	60	7.5	18
	XVI	18.6	65	5.5	18
	XVII	13.7	45	7.6	18
G	XVIII	7.0	40	7.1	18
	XIX	3.5	40	*	18
	XX	0.9	40	*	21
	XXI	5.1	150	†	6

* Disappears very rapidly from the blood † Disappears rapidly from the blood

RESULTS AND DISCUSSION

Compounds have been classified on the basis of their chemical structure into seven groups, each of which is considered separately, the characteristic values obtained from the mean concentration-time curves are given in the Table

Group A 2-Sulphanilamido 4,6-di-n-alkoxypyrimidines



The mean concentrations of these compounds (except II) found in the blood of mice at intervals after the oral administration of 250 mg/kg, are recorded in Fig 1, the characteristic values obtained from the mean curves are compared in the Table. Compounds I-IV are highly persistent, the di-*n*-butoxy homologue (V) is fairly persistent. The other main difference between the five compounds is in maximum blood concentration, which falls with increasing molecular weight, this effect can probably be ascribed mainly to reduced solu-

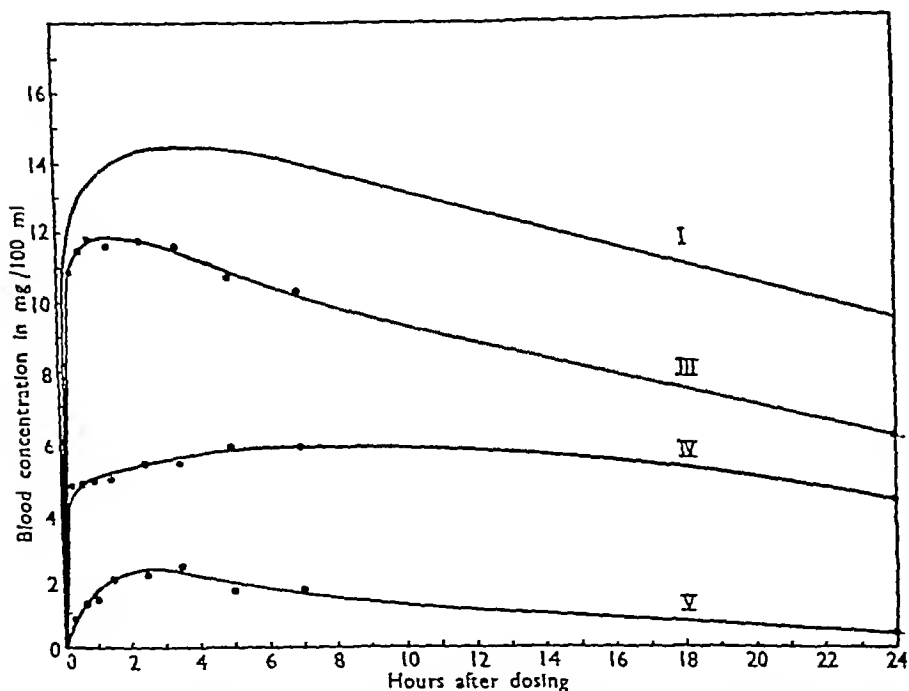
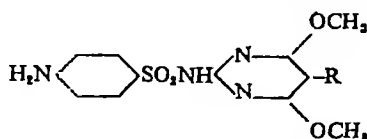


FIG 1—Blood concentrations in mice of sulphadimethoxypyrimidine (I), sulphadiethoxypyrimidine (III), sulphadi-*n* propoxypyrimidine (IV) and sulphadi-*n*-butoxypyrimidine (V)

bility of the higher homologues, which would result in a decreased concentration gradient between lumen and blood stream, and therefore in decreased speed and extent of absorption. The solubility data reported by van Dyke *et al* (1945) for I and III, and an extensive series of related compounds, support this view. The practical importance of the effect is pointed out by Hoggarth *et al* (1948), who show that *in vitro* tuberculostatic activity increases with increasing molecular weight. It is probably the accompanying decrease in blood concentration which prevents the higher homologues from showing marked therapeutic action *in vivo*.

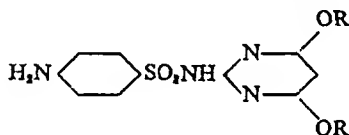
Group B 2-Sulphanilamido-5-alkyl-4,6-dimethoxypyrimidines



Compound No	VI	VII
R	CH ₃	C ₂ H ₅

These two compounds are both highly persistent (see Table), but attain lower maximum blood concentrations than the parent compound. The reduction in maximum concentration is greater than when the additional carbon atoms are substituted in the alkoxy group—i.e., VI and VII give lower concentrations than their isomers II and III. This was unexpected, since in other groups of compounds it has been found that the distribution of a given number of methylene groups between three, instead of two, normal alkyl radicals leads to increased blood concentrations. The effect may be due to the exceptionally low solubility of this type (van Dyke *et al*, 1945, Rose and Tuey, 1946).

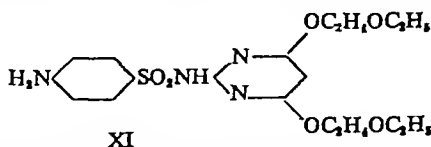
Group C Branched chain sulphadialkoxy derivatives



Compound No	VIII	IX	X
R	<i>iso</i> -C ₃ H ₇	<i>iso</i> -C ₄ H ₉	<i>sec</i> -C ₄ H ₉

The characteristic values of the three compounds are summarized in the Table. They are all highly persistent, the butoxy compounds somewhat less so than the *isopropoxy* compound (cf Group A). VIII and IX resemble their *normal* isomers (IV and V) in maximum blood concentration. X gives a much higher maximum concentration than its isomers V and IX. In some other homologous series, e.g., of sulphones, it has been observed that compounds containing branched alkyl chains give much higher blood concentrations than their isomers containing straight chains. It is hoped to describe examples in future publications.

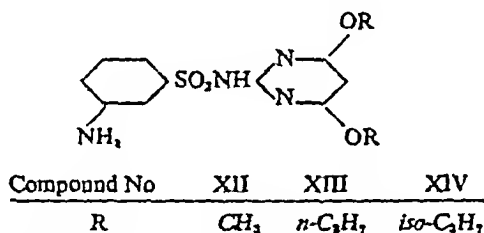
Group D 2-Sulphanilamido-4,6-diethoxyethoxypyrimidine



The ethoxyethoxy compound (see Table) has lost much of the persistence characteristic of the other dialkoxypyrimidines, and gives much higher blood

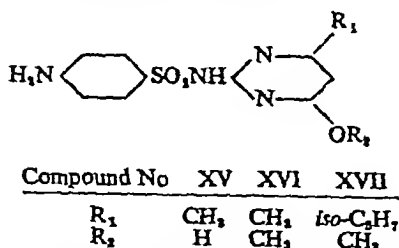
concentrations. It should, of course, be compared with its closest analogue the di-*n*-butoxy derivative (V). The methoxyethoxy homologue was examined by van Dyke *et al*. They found that it was well absorbed, but of low persistence. Its solubility (4 mM/l at pH 6.5 and 37° C) was five times that of the dimethoxy compound (0.8 mM/l), and about thirty times that of the diethoxy compound (0.12 mM/l). It may be assumed that the facile absorption of these alkoxyalkoxy compounds is related to their relatively high solubility, but a different effect must cause the low persistence. The relatively low persistence of the di-*n*-butoxy compound (Group A) suggests that this effect might be connected with steric hindrance, possibly, the bulky alkoxy groups of these compounds hinder their access to some other molecule with which they must be associated to exhibit marked persistence.

Group E 2-Metanilamido-4,6-dialkoxyprymidines



The metanilamides are compared in the Table. Reference should also be made to the properties of the corresponding *para* isomers. Each metanilamide differs from its *para* isomer in giving higher maximum blood concentrations, and in disappearing more rapidly from the blood stream. Nevertheless, all three compounds are fairly persistent, approximately of the same order as sulphamerazine (Rose and Spinks, 1946).

Group F 2-Sulphanilamido-4-alkoxyprymidines



The results with these three compounds are given in the Table. They are all fairly persistent, approximately of the same order as the metanilamides (Group E) and sulphamerazine, but less so than the dialkoxy derivatives. The mean curve for XVII is given in Fig. 2.

Group G Miscellaneous compounds

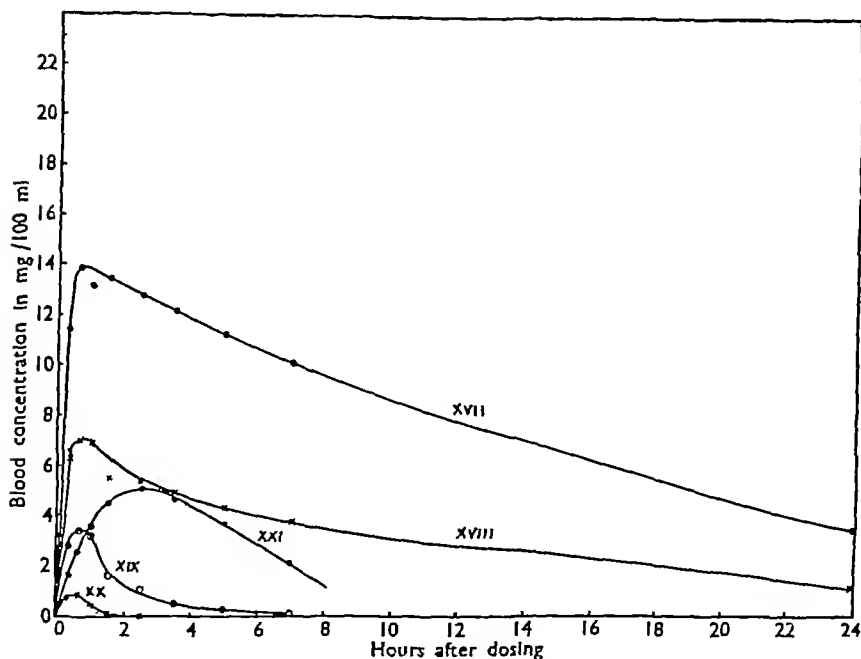
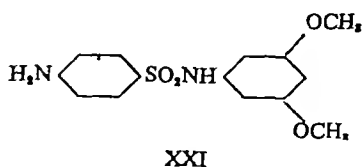
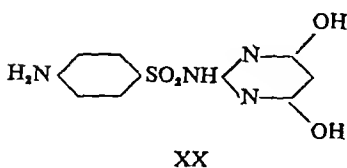
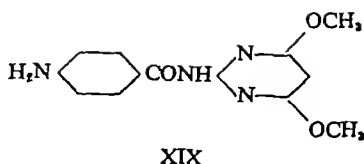
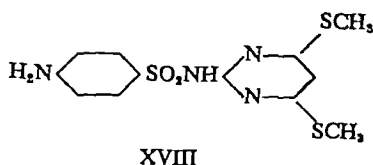


FIG 2—Blood concentrations in mice of sulphamethylisopropoxypyrimidine (XVII) sulphadimethylmercaptopyrimidine (XVIII), 2 *p*-aminobenzamido-4, 6-dimethoxypyrimidine (XIX), sulphadihydroxypyrimidine (XX), and 3, 5-dimethoxy-1-sulphanilamide (XXI)

These compounds show striking differences from those previously described (Fig 2, Table). The dimethylmercapto analogue of sulphadimethoxypyrimidine is less persistent than the latter, and gives a much lower maximum concentration. The other three compounds disappear very rapidly from the blood stream. The low concentrations they attain may be associated with this rapid disappearance.

from the blood rather than with poor absorption. They clearly show no resemblance whatsoever to sulphadimethoxypyrimidine, although each retains certain structural features of the latter.

Considering the results as a whole, it is clear that marked persistence in the blood stream is conferred by the presence in the sulphapyrimidine molecule of two alkoxy groups in positions 4 and 6 of the pyrimidine ring. All modifications of this structure result in reduction of persistence, including removal of one alkoxy group, transfer of the *para* amino group to the *meta* position, substitution of a benzene ring for the pyrimidine ring or substitution of a carboxamide group for the sulphonamide group. The persistence of the dialkoxy compounds appears to fall with increasing molecular weight, although one compound (IV) is a marked exception to this rule.

Although the precise nature of the physical and physiological factors which confer persistence on a compound is at present unknown it seems probable that they include a high degree of binding to the plasma proteins and a high degree of tubular reabsorption (Fisher *et al.*, 1943, Beyer *et al.*, 1944, Earle, 1944, van Dyke *et al.*, 1945). The strength of the bond uniting drug to protein may also be of importance (Gregerson and Rawson, 1943, Rawson, 1943). Sulphadimethoxypyrimidine and some related compounds have been shown to be extensively bound to plasma proteins (van Dyke *et al.*, 1945, Gage *et al.*, 1947), but no information is available on the other important factors. A high degree of protein binding alone would not necessarily result in high persistence, absorption by the tubules being of equal or greater importance (Fisher *et al.*, 1943, Lundquist, 1945). Besides these factors, which influence the rate of excretion of a compound by the kidney, and others, less adequately investigated, which influence the rate of excretion into the intestine (*cf.* Silber and Clark, 1946), diazotizable amines are also removed from the blood stream by conversion to non-diazotizable or rapidly excreted metabolites, such as acetyl derivatives, sulphates or glucuronides. It is improbable that the persistence, even of closely related compounds, is uniformly affected by such metabolic processes.

The complexity of all these mechanisms, which influence persistence, is such that speculation on the nature of the correlation between structure and persistence is hardly justifiable. However, it is clear that the correlation is a very delicate one. It had been hoped that 4,6-dimethoxypyrimidine, or even *m*-dimethoxybenzene, might behave as a "conductophoric" group, and confer persistence on any molecule containing it, in the same manner as the dialkylamino-alkylamino chain of mepacrine, pamaquin, and 3349 (Magidson *et al.*, 1934, 1936, Spinks and Tottey, 1946), or the biguanide chain of paludrine and related drugs (Spinks, 1946, 1947), has been presumed to confer favourable pharmacological properties on the antimalarial containing it. The reduced persistence of compounds XII, XIII, XIV, XIX and XXI strongly suggests that the introduction of 1,3-dimethoxy groups into a nucleus other than pyrimidine, or the combination of

4 6-dimethoxypyrimidine as a "conductophoric" group with a "toxicophoric" group other than sulphanilamide would be unlikely to confer high persistence on the resulting molecule

SUMMARY

The absorption and persistence in mice of twenty compounds related to sulphadimethoxypyrimidine have been described. High persistence is a property of 2-sulphanilamidopyrimidines carrying dialkoxy groups in positions 4 and 6. Removal of one alkoxy group, transfer of the *p*-amino group to the *meta* position, substitution of a benzene ring for the pyrimidine ring, or substitution of a carboxamide group for the sulphonamide group, results in reduced persistence. In each homologous series examined, maximum blood concentration fell with increasing molecular weight.

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